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The geographical distribution of grey wolves in Southern China: misconceptions in western literature

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The geographical distribution of grey wolves (*Canis lupus*) in China: a systematic review

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ABSTRACT

The grey wolf (*Canis lupus*) is one of the most widely distributed terrestrial mammals, and its distribution and ecology in Europe and North America are largely well described. However, the distribution of grey wolves in southern China is still highly controversial. Several well-known western literatures stated that there were no grey wolves in southern China, while the presence of grey wolves across China has been indicated in *A Guide to the Mammals of China*, published by Princeton University Press. It is essential to solve this discrepancy since dogs may have originated from grey wolves in southern China. Therefore, we systematically investigated Chinese literatures about wild animal surveys and identified more than 100 articles and books that included information of the distribution of grey wolves in China. We also surveyed the collections of three Chinese natural museums and found 26 grey wolf skins specimens collected across China. Moreover, we investigated the fossil records in China and identified 25 archaeological sites with wolf remains including south China. In conclusion, with the comprehensive summary of Chinese literatures, museum specimens and fossil records, we demonstrate that grey wolves do distribute across all parts of the Chinese mainland, including the most southern parts.

Keywords: China; Grey wolf; Distribution; Conservation

INTRODUCTION

The grey wolf, *Canis lupus*, is one of the most widely distributed terrestrial mammals (Young & Goldman, 1944). Grey wolves live in a wide variety of habitats, including the dry Arabian desert, the xeric Mediterranean shrublands, the coniferous forests of Siberia, and the frozen tundra on Ellesmere island (Mech, 1981). Despite extirpation from many parts of their previous range over the last few hundred years, by persecution from humans and habitat fragmentation (Hunter & Barrett, 2011; Young & Goldman, 1944), wolves still retain most of their original distributions.

The distribution and ecology of grey wolves are largely well described in Europe and North America. However, in more peripheral and remote parts of their distributions, detailed information is often lacking. In the western literature, the wolf has generally been reported to be distributed throughout the northern hemisphere, from N15° latitude in North America and N12° latitude in India to beyond the Arctic Circle, but has been considered to be absent from Africa and the southern East Asia (Mech, 1981). However, recent articles reported that the Egyptian jackal (*Canis aureus lupaster*, Hemprich and Ehrenberg 1833) was not a subspecies of the golden jackal (*Canis aureus*, Linneaus 1758) and should be reclassified as the African wolf, *Canis lupus lupaster* (Gaubert et al., 2012; Koepfli et al., 2015; Rueness et al., 2001).

Similarly, the literature about wolves in China is limited outside China. This has led to misconceptions in the western literature about the distributions of wolves in

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China. Four studies, all conducted by western researchers, stated that wolf has never been presented in large parts of China (Callaway, 2013; Larson & Fuller, 2014; Nowak, 2003; Sokolov & Rossolimo, 1985).

However, as will be shown in this study the grey wolf has a historical and current range across nearly the entire country of China. There are more than 100 Chinese articles and books involving investigations of wolves in China since the 1950s (Table 1), showing the distributions in detail. Most of these articles are species investigations at a provincial or local level, however, there is no comprehensive description of the current distribution of wolves across China. Therefore, we here summarized the Chinese literature concerning past and present distributions of wolves in China, in order to synthesize data from this rich source of regional investigations into a comprehensive map of wolf distribution in China, and to make this significant information available to an international audience.

Table 1 Literature list of distributions of wolves in China

Province	Investigation year	Location	Reference
Heilongjiang	2008-2009	Eastern forests of Wandashan Mountains	Shen et al., 2011
	1994-2001	Sanjiang National Reserve	Zhang et al., 2001
	1997-1999	Tangwanghe river forest distict	He et al., 2003
	1993-1995	Grand Khingan	Zhang et al., 1998a
	1988-1989, 1993-1995	Northern Grand Khingan	Li et al., 1996
	1984, 1987-1990, 1992	Xingkai Lake Nature Reserve	Li et al., 1993
	1971-1980, 1981-1990	Heilongjiang province	Zhang et al., 1998b
	N/A	Heilongjiang province	Zhang & Yu, 2005
	N/A	Western Helongjiang province	Gao et al., 1999
Jilin	1992-1993	Yanbian	Qiu et al., 1995
	1992-1993	Hunjiang	Li et al., 1994
Liaoning	1999-2002	Nuluerhusan National Reserve	Zhou et al., 2007
	1996-2000	Benxi	Zhao et al., 2004a
	1996-2000	37 counties in Liaoning province	Zhao et al., 2004b
	1996-1999	Fushun	Zhao et al., 2001
	N/A	Yiwulv Mountain National Nature Reserve	Liu et al., 2008
	N/A	Liaoyang	Wang et al., 2004
Inner Mongolia	1985-1986	Jiufeng Shan	Liu & Liu, 1999
	N/A	Chaihe	Xiao et al., 2013
	N/A	Hulunbair & Hinggan	Gao et al., 1999
Beijing	1982-1983	Changping & Miyun	Zhang, 1984
	N/A	Beijing	Wu et al., 2006
Tianjin	N/A	Tianjin	Wu et al., 2006
Shanxi	2010-2011	Pangquangou National Nature Reserve	Wang & Zhao, 2011
	1996-1997	Luyashan Nature Reserve	Qiu et al., 1998
	N/A	Northeastern Loess Plateau	Chen, 2000
Hebei	1993-2001	Chengde	Hou et al., 2004
	N/A	Hebei province	Wu et al., 2006
	N/A	Saihanba	Hou et al., 1994
Gansu	2007-2009	Sunan and Subei prairie	Zhao et al., 2011
	N/A	Gannan plateau	Chen & Li, 1994
	N/A	Longnan mountain	Chen et al., 1994
	N/A	Tianshui	Hu et al., 1993
	N/A	Minqin desert	Chen, 1992
	N/A	Anxi	Chen & Luo, 1991

Continued

Province	Investigation year	Location	Reference
Xinjiang	1994-1996	Kanas National Nature Reserve	Abdukadi et al., 1999
	1987-1988	Wuqia, Taxkorgan, Yecheng, Qiemo, Yutian	Feng, 1990
	1965, 1980, 1983, 1985	Zhungeer & Altai	Zhang & Hu, 1988
	1979	Xinjiang	Gao, 1997b
	1958-1961	Desert plains area in Xinjiang	Zhang, 1963
	N/A	West Tianshan National Nature Reserve	Liu et al., 2007a
Ningxia	2010-2011	Luoshan National Nature Reserve	Qin & Chang, 2012
Shaanxi	2006	Huanglongshan Nature Reserve	Li & Liu, 2009
	2006	Micangshan Nature Reserve	Wen et al., 2008
	1997-2000	Changqing National Nature Reserve	He, 2001
	1999	Zhashui	Hu et al., 2003
	1996	Zhouzhi National Nature Reserve	Li & He, 1997
	1963-1966	Ankang	Wu & Li, 1982
	1959	Daba mountain	Wang et al., 1981
	N/A	Shaanxi province	Li et al., 2006
Qinghai	2001-2002	Qilian mountain	Xia et al., 2003
	N/A	Beichuan River Nature Reserve	Zhang & Pu, 2012
	N/A	Qinghai lake area	Kong et al., 2011
Tibet	2001-2002	Upper Zayu river basin	Wu, 2006
	1987-1988	Ngari & Naqu	Feng, 1990
Sichuan	2006	Kasha Lake Nature Reserve	Liu et al., 2013
	1997, 2006	Ruoergai Wetland National Nature Reserve	Liu et al., 2009
	2005-2006	Maozhai Nature Reserve	Liu et al., 2007b
	2003-2005	Haizishan Nature Reserve	Liu et al., 2007c
	2004	Heizhugou Nature Reserve	Liu et al., 2005a
	2002-2003	Jiuzhaigou National Nature Reserve	Liu et al., 2005b
	2002-2003	Dafengding Nature Reserve	Liu et al., 2004
	2002-2003	Yele Nature Reserve	Zhang & Hu, 2004
	2001-2002	Huanglong Nature Reserve	Zhu et al., 2010
	2002	Xuebaoding Nature Reserve	Sun et al., 2006
	2001	Pingwu	He et al., 2004
	1998	Big-small Langou Nature Reserve	Lu & Hu, 2003
	1996	Huanglongsi Nature Reserve	Hu et al., 2001
	N/A	Ganzi and Liangshan	Zhang et al., 2009
	N/A	Ruoergai Wetland National Nature Reserve	Hao et al., 2008
	N/A	Wolong Nature Reserve	Yu et al., 1983
Yunnan	2010-2011	Lanping Yunling Provincial Nature Reserve	Cui et al., 2014
	2010-2011	Weixi	Zha et al., 2014
	N/A	Yunnan province	Yang et al., 1999
Guizhou	2005-2006	Leigong Mountain National Nature Reserve	Chen et al., 2008
	N/A	Guizhou province	Luo & Li, 2001
	N/A	Weining	Huang, 1989

Continued

Province	Investigation year	Location	Reference
Chongqing	2006-2008	Jinfo Mountain Natural Reserve	Zong et al., 2010
	1995	Jinfo Mountain Natural Reserve	Peng et al., 1996
	N/A	Chongqing	Han & Hu, 2002
Henan	1997	Xin'an, Yuzhou, Jiyuan, Luoning, Jiaozuo, Zhenping	Gan & Fan, 2004
Hubei	2004	Yerengu Nature Reserve	Wang et al., 2007
	2004	Wudaoxia Nature Reserve	Wu et al., 2005
	2001	Qizimei Mountain Nature Reserve	Liu et al., 2002
	N/A	Duheyuan Provincal Nature Reserve	Li et al., 2008
Hunan	1980-1981	Ziyunshan	Fu, 1987
Jiangxi	2004-2007	Taohong Ridge Sika Deer Nature Reserve	Wu et al., 2012
	1984-1986	Poyang lake area	Fu & Ding, 1991
	N/A	Jiangxi province	Tu et al., 2014
	N/A	Lushan Nature Reserve	Li et al., 2007
Shandong	1984-1987	Jiaodong peninsula	Sun, 1988
	1982-1986	Qingzhou	Cong, 1988
	1961-1966, 1973-1984	Jiaodong and Luzhongnan area	Lu, 1984
	N/A	Laoshan	Tian et al., 2000
Anhui	1959-1964	Anhui province	Wang et al., 1966
	N/A	Anhui province	Wu et al., 2002
	N/A	Huangshan	Xu, 1997
Jiangsu	N/A	Jiangsu province	Wang & Zhao, 2008
Zhejiang	2005-2008	Hangzhou	Ding et al., 2008
	1958-1960, 1962-1964, 1979-1981	Zhejiang province	Zhuge, 1982
	N/A	Jinhua	Zhu & Yu, 1996
	N/A	Yongkang	Bao & Hu, 1987
Fujian	N/A	Fujian province	Chen et al., 2009
	N/A	Fujian province	Zhou, 1997
	N/A	Fujian province	Zhan, 1995
Guangxi	1997-2000	Shiwan Mountain	Xia et al., 2002
	1958	Southwestern Guangxi	Wang et al., 1962
Guangdong	2000	Nanling National Nature Reserve	Fellowes et al., 2003

LITERATURE SUMMARIZATION

It is controversial to describe the distribution of grey wolves in western literatures. Two articles reported that wolves were previously present all across China, but is now extinct from southern China (Ginsberg & Macdonald, 1990; Lau et al., 2010). In four well-known studies, researchers claimed that wolves have never existed in sourthern China (Callaway, 2013; Larson & Fuller, 2014; Nowak, 2003; Sokolov & Rossolimo, 1985), suggesting that sourthern China cannot be the harbor of dog domication. Thus, southern China is usually treated outside the range of wolf distribution (IUCN; EOL). However, in 2008, Smith and his colleagues described the distribution of wolf in China, indicating that grey wolves were present all across the mainland of China (Smith & Xie, 2008).

In the Chinese literature, wolves have been reported to appear over all parts of continental China. The Fauna Sinica (China): Mammalia Vol. 8 Carnivora page 46-49, reported in 1987: “the wolf, which apart from Hainan Island, the various islands in the South China Sea, and Taiwan, is spread over nearly all the country“ and ”the wolf can be seen in all provinces. Based on collected literature references and specimen samples, wolves have been identified in Muleng, Baoqing, and Genhe of Heilongjiang, in Baicheng, Kaitong, Dunhua, Jingyu, Huinan, Hunchun, Jilin, Tumenling, and Fuyu of Jilin, in Fushun and Lvda of Liaoning, in Shanhaiguan and Zhangjiakou of Hebei, in Beijing, in Hohhot and Erlian of Inner Mongolia, in Hami, Bole, Turpan,

Yanqi, Korla, Aksu, Luntai, and Baicheng of Xinjiang, In Shanxi province, in Yan'an of Shaanxi, in Mianchi and Luoning of Henan, in Yichang of Hubei, in Nanjing and Qingjiang of Jiangsu, in Fujian province, in Longzhou, Ningming, and Shangsi of Guangxi, in Guangdong province, in Guizhou province, in Lushui and Chengkou of Yunnan, in Yumen, Zhangye, and Linxia of Gansu, in Menyuan, Qilian, Alaer, Golmud, and Delingha of Qinghai, in Pali, Nylamu, Tingri, Shigatse, and Naqu of Tibet, and in Shiqu, Ruoergai, Songpan, Leibo, Ebian, Kangding, Wanxian, Yibin, and Mianyang of Sichuan" (Gao & Wang, 1987).

Furthermore, Wang (2003) described the subspecies/subtypes of grey wolvesin China and reported that they were distributed across all parts of continental China. Chinese wolves were divided into five subspecies and forms: *Canis lupus desertorum* Bogdanow, 1882 in Xinjiang, *C. l. filchneri* Matschie, 1907 in Qinghai, Gansu and Tibet, *C. l. chanco* Gray, 1863 in Heilongjiang, Jilin, Liaoning, Inner Mongolia (eastern part), Hebei, Beijing, Shandong, Henan and Shanxi, *C. l.* Nei-Mongol form in Inner Mongolia (western and mid part) and *C. l.* South-China form in Anhui, Jiangsu, Zhejiang, Jiangxi, Fujian, Guangdong, Hunan, Guizhou, Yunnan, Hubei and Sichuan

In order to obtain an updated and comprehensive description of the distribution of wolves in China, we investigated more than 100 articles containing information about the presence of wolf at a regional level (see a full list of literature in Table 1). The most recent evidence of wolf in each province (Figure 1) were extracted from the following papers: Heilongjiang (Shen et al., 2011), Jilin (Qiu et al., 1995), Liaoning (Zhou et al., 2007), Inner Mongolia (Liu & Liu, 1999), Beijing (Zhang, 1984), Tianjin (Wu et al., 2006), Shanxi (Wang & Zhao, 2011), Hebei (Hou et al., 2004), Gansu (Zhao et al., 2011), Xinjiang (Abdukadir et al., 1999), Ningxia (Qin & Chang, 2012), Shaanxi (Li & Liu, 2009), Qinghai (Xia et al., 2003), Tibet (Wu, 2006), Sichuan (Liu et al., 2013), Yunnan (Cui et al., 2014), Guizhou (Chen et al., 2008), Chongqing (Han et al., 2010), Henan (Gan & Fan, 2004), Hubei (Wang et al., 2007), Hunan (Fu, 1987), Jiangxi (Wu et al., 2012), Shandong (Sun, 1988), Anhui (Wang et al., 1966), Jiangsu (Wang & Zhao, 2008), Zhejiang (Ding et al., 2008), Fujian (Chen et al., 2009), Guangxi (Xia et al., 2002), Guangdong (Fellowes et al., 2003).

Figure 1 Distributions of wolves in China

The latest investigation year recorded in literature in 26 provinces (in red) and the latest publication year of literature in three provinces (in green) are indicated within brackets.

In summary, these investigations showed that the wolf has been recorded in every continental Chinese province between 1964 and the present, except in three provinces (Figure 1 in green). Most notably, wolves were recorded in South China (in Yunnan province) as late as 2011 and in the two southernmost continental provinces (Guangdong and Guangxi) in the year of 2000. From these findings we concluded that wolves are still present across all parts of continental China.

WOLF SKINS IN ZOOLOGICAL MUSEUMS

In addition to the literature investigation, we made a survey of wolf skins in the archives of the National Zoological Museum of China, Kunming Natural History Museum of Zoology, and Shaanxi Institute of Zoology, and (Table 2, Figure 2, Figure 3). We found 26 wolf skins sampled from 13 provinces across China, e.g., two specimens sampled from two southern Chinese provinces (Zhejiang and Fujian) in 1974, and one from southern Yunnan in 1985.

WOLF FOSSIL RECORD

We investigated the literature about archaeological research in China, to identify information about wolf fossils in archaeological sites. We extracted information about the fossil record of the grey wolf in China from three Chinese books (Lv, 2004; Yuan, 2015; Zhang et al., 2003). These books reported 25 archaeological sites in 14 provinces across China with wolf fossils records (Table 3), including the 12 000 years old remains from the South Chinese province Jiangxi.

Table 2 Sources and geographical origins of wolf skin specimens

Museum	ID	Province	Location	Date
The National Zoological Museum of China, Beijing	1	Heilongjiang	Baoqing	N/A
	2	Heilongjiang	Baoqing	1957.01.24
	3	Inner Mongolia	Xiguitu (Yakeshi)	1954.12.10
	4	Jilin	Baicheng	1957.02.11
	5	Jilin	Jingyu	1956.03.08
	6	Jilin	Kaitong	1956.06.13
	7	Xinjiang	Buerjin	1974
	8	Xinjiang	Bole	1972.05.18
	9	Tibet	N/A	N/A
	10	Tibet	Changdu	1976.1
	11	Tibet	N/A	N/A
	12	Beijing	Yanqing	1984.04.28
	13	Sichuan	Ruo'ergai	1961.07.03
	14	Yunnan	Lushui	1960
	15	Fujian	N/A	1974.05
	16	Zhejiang	Lin'an	1974
Kunming Natural History Museum of Zoology, Kunming	17	Yunnan	Kunming	1967
	18	Yunnan	Kunming	1957
	19	Yunnan	Zhaotong	N/A
	20	Yunnan	Honghe	1985
	21	Guizhou	N/A	N/A
	22	Guizhou	N/A	N/A
	23	Jiangxi	Zoo	1990.06.08
Shaanxi Institute of Zoology, Northwest Institute of Endangered Zoological Species, Xi'an	24	Shaanxi	Yan'an	1973
	25	Shaanxi	Xunyang	1965
	26	Shaanxi	Pingli	1965

DISCUSSION

In this study, we showed that contrary to what is reported in many references in the western literature, the grey wolf actually is present across virtually all parts of the mainland China. This correction is important in studies of wolf ecology and conservation. It gives a correct picture of the worldwide

Figure 2 Source and geographical origin of museum wolf skin specimens

distributions of wolves, by filling in a large blank region on the map. It is also important in studies of the history of domestic dogs, since dogs probably trace a large proportion of their genetic ancestry to wolves from the southern parts of East Asia (Wang et al., 2016).

The wolf has endured massive decline in population size and geographic range around the world during the previous two centuries, because of human influence including habitat loss, persecution, hunting (for obtaining, e.g., trophies, furs and material for traditional medicine), and depletion of prey (Beschta & Ripple, 2010; Callan et al., 2013; Levi & Wilmers, 2012; Ripple et al., 2014). Also in China, the distribution areas of wolves have severely decreased due to human mediated habitat loss and hunting (Gao, 1997a, 2006; Zhang, 1999). Official investigations from the middle of the 20th century reported that wolves were distributed in every province of China except some islands, but gave no exact numbers. Today, large populations remain only in the northwestern and northeastern parts of the country, Inner Mongolia and Tibet, but even in these regions, the numbers are relatively small, e.g., only 2 000 wolves in Inner Mongolia were reported in the 1990s (Gao, 1997a). We have here shown that wolves still seem to be present across all parts of the Chinese mainland, including the most southern provinces. Thus, even though habitat loss has been severe in urban and agricultural regions, wolves seem to have persisted in intervening regions.

The data about wolf distributions that we here present were investigations on either provincial or local level, whereas, a comprehensive ecological survey of the wolves in China. It is therefore not clear how the wolf populations in the different parts of China are interrelated. For example, it is not clear whether wolves recorded in the southern provinces represent permanent populations, or a steady stream of individuals migrating from the northern provinces. However, it is notable

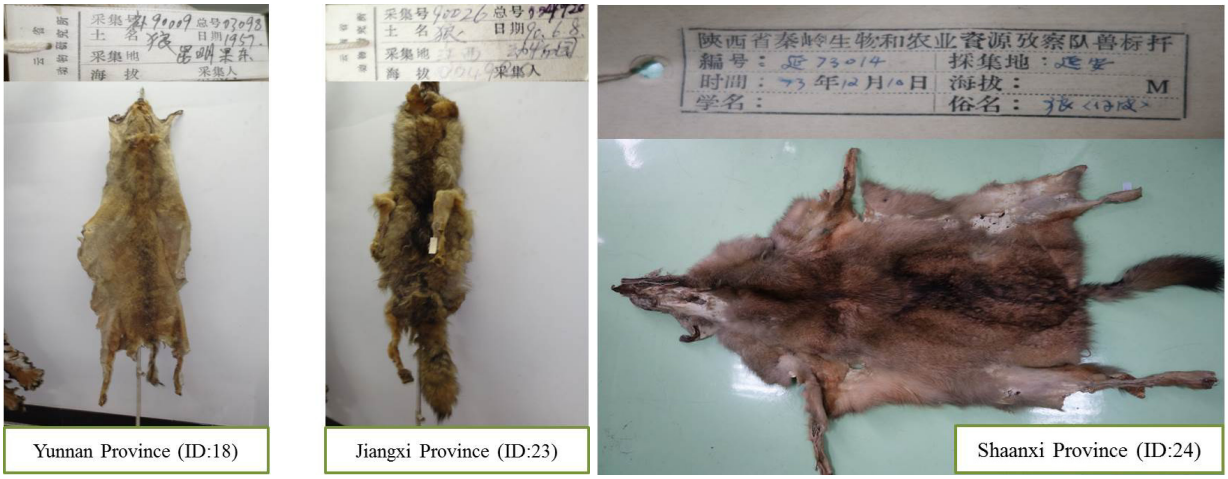

Figure 3 Three museum wolf skin specimens

Specimens originating from Yunnan Province (left, ID 18 in Table 2), Jiangxi Province (middle, ID 23 in Table 2) and Shaanxi Province (right, ID 24 in Table 2).

Table 3 Fossil records of gray wolves

Province	County	Archaeological site	Time	Reference	
Shanxi and Hebei	Yanggao and Yangyuan	Xujiayao	About 100 000 years ago	Zhang et al., 2003	p259
Shaanxi	Pucheng	Nanwan and Beiwan	Epipleistocene		p315
Henan	Anyang	Xiaonanhai	22 150-11 000 years ago		p320
Heilongjiang	Harbin	Yanjiagang	22 370±300 years ago		p357
Shanxi and Hebei	Yanggao and Yangyuan	Xujiayao	125 000-104 000 years ago	Lv, 2004	p96
Hebei	Yangyuan	Banjing	108 000-74 000 years ago		p100
Shanxi	Yanggao	Shenquansi	11 720±150 years ago		p102
Liaoning	Haicheng	Xiaogushan	Epipleistocene		p207
Chongqing	Fengjie	Yufupu	7 560±110 years ago		p355
Heilongjiang	Mishan	Xinkailiu	7 500-6 500 years ago	Yuan, 2015	p114
	Qiqihar	Tengjiagang	Bronze age		p115
	Hailin	Xilinhe	Bohai Kingdom (698-926 A.D.)		p115
Jilin	Nong'an	Zuojiashan	6 800-4 800 years ago		p115
Liaoning	Dalian	Guojiacun	5 780-4 300 years ago		P118
Inner Mongolia	Linxi	Baiyingchanghan	8 000-5 000 years ago		P120
	Baotou	Yanjialiang	1 275-1 372 years ago		p127
Shaanxi	Nanzheng	Longgangsi	6 500-6 000 years ago		p130
	Tongchuan	Beicun	Shang Dynasty (1 600-1 046 B.C.)		p133
Hebei	Xushui	Nanzhuangtou	About 10 000 years ago		p144
Beijing	Fangshan	Zhenjiangying and Tazhao	Shang and Zhou Dynasties (1 600-256 B.C.)		p145
Shandong	Yanzhou	Wangyin	6 500-5 500 years ago		p147
	Weifang	Qianbuxia	Houli Culture (8 500-7 500 years ago) and 5 500-5 000 years ago		p147
Tibet	Naqu	Chaxiutang	9th-11th century A.D.		p155
Hubei	Zigui	Liulinxi	Neolithic age, Erlihe Culture (21st-15th century B.C.), and the Eastern Zhou Dynasty (770-256 B.C.)		p158
	Badong	Lijiatuo	Eastern Zhou Dynasty (770-256 B.C.)		p164
Jiangxi	Wannian	Xianrendong	About 12 000 years ago		p166

that wolves have been recorded across virtually the entire continental China, including southern Chinese province Yunnan as late as in 2011 and provinces Guangdong and Guangzhou in 2000. These findings indicate a consistent presence of permanent populations across southern China. Moreover, to obtain a comprehensive picture of the status of the wolves in China, it is necessary to carry out both ecological and genetic studies, e.g., in concerning the genetic relationships either among the wolf populations across China and between these and worldwide wolf populations.

This study points out misconceptions in the western literature about the distributions of wolves in China. The origin of this problem is not clear, but it can be traced back as far as an article in 1985 from which the factoid has, stepwise, been passed on to other articles (Sokolov & Rossolimo, 1985). It is probably because of the linguistic barrier to the Chinese literature that this error has previously not been pointed out. This case can be explained by inefficient research in peripheral parts of the species distribution, in countries with limited resources. Our study raises the question whether this kind of misconceptions also exist in other species than just the grey wolf.

CONCLUSIONS

With a comprehensive summary of Chinese literature, specimens and fossil records, we showed that wolves are present across all parts of the Chinese mainland, including the southern parts. Hereby we corrected an error in western literature, in which most sources stated that wolves were not present in the southern China, and some even claimed that wolves have never been presented there, even in ancient times. There is no comprehensive description of the current distributions of wolves across China, and therefore this study serves both to give an updated description of wolf distributions in China, and to make this significant information available to an international audience.

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A new species of genus *Fejervarya* (Anura: Dicroglossidae) from northern Thailand

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ABSTRACT

We describe a new species of frog in the dicroglossid genus *Fejervarya* from Ban Monjong, Omkoi District, Chiang Mai Province, northern Thailand. Analysis of DNA sequence data from the mitochondrial gene 16S, advertisement calls, and morphological distinctiveness support recognition of the new species. Matrilineal genealogy suggests that the new population from Chiang Mai is a sister taxon to the South Asian clade that includes *F. syhadrensis, F. granosa*, and *F. pierrei*. The new species, *Fejervarya chiangmaiensis* **sp. nov.**, differs morphologically from its congeners by its relatively small body size and proportions and the presence of dorsal warts and dermal ridges. Discovery of this new species indicates that the biodiversity of amphibians in this region remains underestimated.

Keywords: Phylogeny; Mitochondrial DNA; 16S rRNA; Chiang Mai Province; Cryptic species; *Fejervarya chiangmaiensis* **sp. nov.**

INTRODUCTION

The cricket frogs *Fejervarya* Bolkay currently contain 40 species (Frost, 2016), most of which occur in East and Southeast Asia, to the Indian subcontinent, including Sri Lanka, and further to Pakistan, Nepal, and Bangladesh (Dinesh et al., 2015). This genus comprises two reciprocally monophyletic species groups: (1) South Asian group; and (2) East and Southeast Asian group (Dinesh et al., 2015). Seven species within these two groups occur in Thailand (Frost, 2016), including *Fejervarya andamanensis* (Stoliczk, 1870); *Fejervarya cancrivora* (Gravenhorst, 1829); *Fejervarya limnocharis* (Gravenhorst, 1829); *Fejervarya moodiei* (Taylor, 1920); *Fejervarya multistriata* (Hallowell, 1861); *Fejervarya orissaensis* (Dutta, 1997); and, *Fejervarya triora* (Stuart et al., 2006). Except for *F. andamanensis*, which assigns to the South Asian group, all other Thai species assign to the East and Southeast Asian group (Frost, 2016). Several molecular studies have suggested that *F. limnocharis* from this region might represent an unnamed species (Dinesh et al., 2015; Kotaki et al., 2010; Kuramoto et al., 2007), such as, *F.* sp. hp3 from Pilok, Thailand

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and *F.* sp. hp2 from Bankok, Thailand (Kotaki et al., 2010). However, morphological characteristics of specimens from these regions have not been examined in detail. A closer inspection of many of these species is necessary to better understand and effectively manage the amphibian biodiversity in Thailand.

Our recent fieldwork in Thailand resulted in the discovery of a new population of *Fejervarya*. To clarify its phylogenetic relationships with other species of *Fejervarya*, we reconstructed a matrilineal genealogy for the genus using mitochondrial DNA (mtDNA) sequence data from the ribosomal RNA gene 16S, which is widely recognized as a useful genetic marker for amphibian systematics (Vences et al., 2005a, b). Our genealogy provides evidence for the phylogenetic placement of the new species. In addition, we examined the major morphological characters and acoustic data traditionally used in dicroglossid frogs. Based on these data, we describe the population as a new species of *Fejervarya*.

MATERIALS AND METHODS

Sampling

Fieldwork was conducted in the vicinity of Monjong village, Omkoi District, Chiang Mai Province, Thailand (Figure 1) from June to September 2013. Twelve adult male frogs were collected in the field and photographed *in situ*. Specimens were euthanized using benzocaine after extraction of liver tissue, which was stored in 95% ethanol. The voucher specimens were fixed with 10% formalin and later stored in 70% ethanol. All specimens were deposited in the herpetological collection of the Museum of the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). The protocols for collection of specimens in this study were approved by the Animal Ethics Committee of the KIZ, CAS.

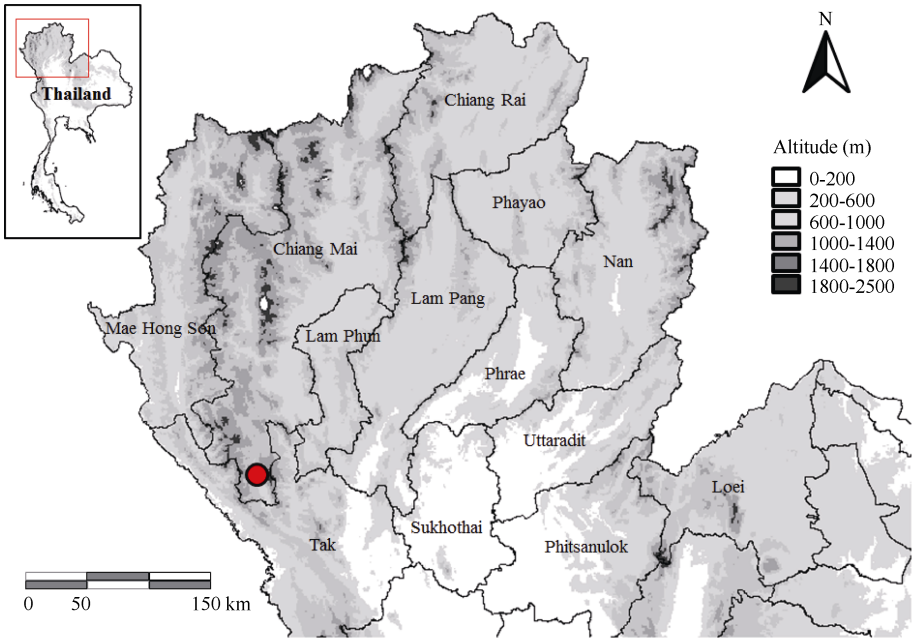

Figure 1 Known distribution of *Fejervarya chiangmaiensis* sp. nov. from northern Thailand, Omkoi District, Chiang Mai Province (red cycle=type locality)

Molecular methods

Total genomic DNA was extracted from tissue samples using standard phenol-chloroform protocols (Sambrook et al., 1989). One fragment of mtDNA encompassing the 16S rRNA gene (16S) was amplified using primers 16Sar: 5'-CGCCTGTTTAYC AAAAACAT-3' and 16Sbr: 5'-CCGGTYTGAACTCAGATCAY GT-3' from Kocher et al. (1989). Amplification was performed in a 25 μL volume reaction with the following procedure: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were purified with a Gel Extraction Mini Kit (Watson Biotechnologies, Shanghai, China). All sequencing was conducted on a ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) at KIZ, CAS. All individuals were sequenced in both directions.

Phylogenetic analysis

Sequences were examined for quality of signal and confirmed for complementarity using DNASTAR 5.0 (DNASTAR Inc., Madison, WI, USA). As the very small body size of the new specimens was most similar to that of species from the South Asian *Fejervarya* group, we chose *F. limnocharis*, *F. triora*, and *Limnonectes fujianensis* as outgroups based on Kotaki et al. (2008, 2010). Available sequences for these species were downloaded from GenBank as outgroups (Table 1). All mtDNA sequences were aligned using MUSCLE (Edgar, 2004).

Table 1 Specimens corresponding to genetic samples included in phylogenetic analysis, their localities, and GenBank accession numbers

Species	Museum Cat. No.	Locality	GenBank No.	Source
Ingroup				
F. chiangmaiensis **sp. nov.**	KIZ024057	Thailand: Chiang Mai; Omkoi	KX834135	This study
F. chiangmaiensis **sp. nov.**	KIZ024126	Thailand: Chiang Mai; Omkoi	KX834136	This study
F. sahyadris	RBRL 050714-02	India: Aralam	AB530605	Hasan et al. (2014)
F. syhadrensis	-	Sri Lanka	AY141843	Meegaskumbura et al. (2002)
F. gomantaki	CESF 2295	India: Goa	KR78086	Dinesh et al. (2015)
F. granosa	-	India: Mudigere	AB488895	Kotaki et al. (2010)
F. pierrei	-	Nepal: Chitwan	AB488888	Kotaki et al. (2010)
F. kudremukhensis	-	India: Kudremukh	AB488898	Kotaki et al. (2010)
F. cf. *nilagirica*	-	India: Western Ghats; Kudremukh	AB167949	Kurabayashi et al. (2005)
F. cf. *syhadrensis*	-	India: Karnool	AB488893	Kotaki et al. (2010)
F. caperata	-	India: Mudigere	AB488894	Kotaki et al. (2010)
F. greenii	-	Sri Lanka: Hakgala	AB488891	Kotaki et al. (2010)
F. kirtisinghei	MNHN 2000.620	Sri Lanka: Laggalla	AY014380	Kosuch et al. (2001)
F. rufescens	030526-03	India: Western Ghats; Mangalore	AB167945	Kurabayashi et al. (2005)
F. cf. *brevipalmata*	030607-01	India: Western Ghats, Madikeri	AB167946	Kurabayashi et al. (2005)
F. mudduraja	-	India: Madikeri	AB488896	Kotaki et al. (2010)
F. keralensis	WII:3263	India	JX573181	Unpublished
F. limnocharis	-	Indonesia: Java	AB277302	Kotaki et al. (2008)
F. triora	-	Thailand: Ubon Ratchatani	AB488883	Kotaki et al. (2010)
Outgroup				
Limnonectes fujianensis	-	China	AF315152	Unpublished

"-" denotes no museum Cat. No.

Phylogenetic reconstructions were executed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Character-based MP analyses were conducted using PAUP* v4.0b10 (Swofford, 2003). Full heuristic tree searches with tree bisection-reconnection were executed for 1000 replications. Bootstrap support (BS) for the MP tree involved 1 000 pseudoreplicates (Felsenstein, 1985). The ML analyses were performed with RAxML v7.0.4 (Stamatakis et al., 2008) using the Gamma model of rate heterogeneity option. Nodal support was estimated using 1 000 BS pseudoreplicates. For BI, the best-fit model of DNA sequence evolution was chosen using MrModeltest v2.3 (Nylander, 2004) under the Akaike information criterion. The GTR+I+G model was selected and used to generate a BI tree using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Analyses were run for five million generations using four chains while sampling one of every 1000 tree generations and discarding the first 25% as burn-in. Log-likelihood scores were tracked to assure stationarity.

Genetic distances among taxa were calculated using the Kimura 2-parameter model in MEGA 5. The matrilineal genealogy was assumed to reflect the phylogenetic relationships of the species. We considered tree nodes with bootstrap values 70% or greater and posterior probabilities values over 0.95 as sufficiently resolved, those between 75% and 50% (0.95 and 0.90 for BI) as tendencies, and those below 50% (0.90 for BI) as non-resolved (Huelsenbeck & Hillis, 1993).

Morphology

Measurements were made with digital calipers to the nearest 0.1 mm. Twenty morphometric characters of post metamorphic individuals were as per Matsui (1984) as follows: (1) snout-vent length (SVL); (2) head length (HL); (3) snout-nostril length (S-NL); (4) nostril-eye length (N-EL); (5) snout length (SL); (6) eye length (EL); (7) tympanum-eye distance (T-ED); (8) head width (HW); (9) internarial distance (IND); (10) interorbital distance (IOD); (11) upper eyelid width (UEW); (12) forelimb length (FLL); (13) lower arm length (LAL); (14) first finger length (FFL); (15) hindlimb length (HLL); (16) tibia length (TL); (17) foot length (FL); and (18) inner metatar sal tubercle length (IMTL). Additionally, we also measured (19) finger length (I-IV FL) and (20) toe length (I-V TOEL). Toe-webbing states followed Savage (1975).

We obtained comparative morphological data from museum specimens (Table 3), photographs of these specimens in life,

and previously published literature: Jerdon, 1853; Günther, 1859, 1869; Peters, 1871; Boulenger, 1905; Annandale, 1919; Rao, 1922, 1937; Smith, 1930; Taylor, 1962; Inger, 1966; Dubois, 1975, 1984; Pillai, 1979; Manamendra-Arachchi & Gabadage, 1996; Dutta, 1997; Manthey & Grossmann, 1997; Dubois et al., 2001; Stuart et al., 2006; Orlov & Ananjeva, 2007; Matsui et al., 2007; Kuramoto et al., 2007; Ohler et al., 2009; Djong et al., 2011; Howlader, 2011a, b; and Purkayastha & Matsui, 2012. Due to the high level of cryptic diversity within *Fejervarya*, we relied on examination of topotypic material and/or original descriptions of species when available.

Acoustics

Advertisement calls of the newly collected population were recorded *in situ* on 30 June, 2013, from 2200h to 2330h using a digital recorder (EDIROL R-09, Roland, Swansea, UK) with built-in microphone (frequency responses of 20–22.000 Hz). Files were recorded as 16-bit WAV files at a sampling frequency of 44.1 kHz and 22 advertisement calls from a single individual were recorded. The ambient temperature (26 °C) was measured with a digital thermometer immediately after recording. Total duration of the recording was 3.0 s. We generated sound spectrograms of all field recordings using Syrinx-PC sound analysis software (J. Burt, Seattle, WA, USA) with the following settings: FFT size 512 samples and Hanning FFT window for spectrograms and power spectra, with FFT samples overlapping 75% for spectrograms. Comparative advertisement call characters for dicroglossids were taken from Kuramoto et al. (2007), Ohler et al. (2009), and Purkayastha & Matsui (2012).

RESULTS AND DISCUSSION

Morphological measurements and variations are summarized in Table 3. This species had both short and long calls, though the latter were not always emitted (Figure 2). The shorter call consisted of a series of pulsed notes. Each of these notes lasted 3.0±0.4 s and was composed of 9-12 pulses/call (average 11.2±1.8). The note interval was 1.81±0.598 s, the dominant frequency was 2.0±0.03 kHz, and the second harmonic was about 3 857±0.036 kHz. The call had a slight frequency modulation.

All unique *de novo* sequences were deposited in GenBank under accession numbers KX834135 and KX834136. Sequencing generated a total of 700 base pairs (bp) of 16S rRNA data, among which 496 positions were potentially parsimony-informative. The ML, MP, and BI analyses produced similar topologies. Monophyly of the South Asian group of *Fejervarya* was recovered (Figure 3). Within these frogs, 16 species occurred in strongly supported matrilines A and B (Figure 3). Matriline A contained seven matrilines A1-7 (Figure 3), but their relationships were generally poorly resolved. Sub-matriline A1 contained the new population from Chiang Mai, which was the sister group of sub-matriline A2, which included *F. syhadrensis*, *F. granosa*, and *F. pierrei*. *Fejervarya sahyadris* formed a sister relationship with *F.* cf. *syhadrensis* within sub-matriline A3. Sub-matriline A4 included *F. kudremukhensis* and *F.* cf. *nilagirica*. Sub-matriline A5 included *F. caperata*. Sub-matriline A6 included *F. greenii* and *F. kirtisinghei*. Sub-matriline A7 included *F. rufescens*. Matriline B was comprised of *F. brevipalmata*, *F. murthii*, and *F. keralensis*.

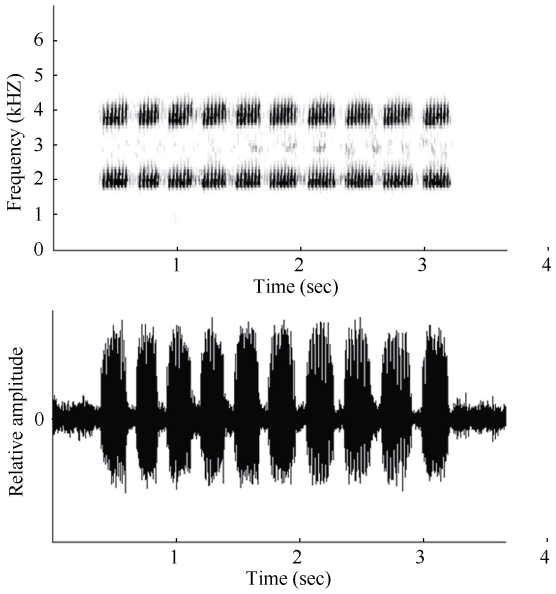

Figure 2 Sonogram and oscillogram of a *Fejervarya chiangmaiensis* sp. nov. call recorded on 30 June, 2013, at an agricultural farm in Ban Monjong, Omkoi District, Chiang Mai Province, Thailand

The genetic distances among the 13 South Asian species ranged from 0.4% to 14.8% (Table 2). Interspecific genetic distances between the newly discovered Chiang Mai matriline A1 and members of sub-matriline A2 from Sri Lanka, India, and Nepal ranged from 6.0% to 6.7%. The new species differed from *F.* cf. *syhadrensis* and *F. sahyadris* (both within matriline A2) from India by 8.8% and 9.0%, respectively. The new species differed from *F. caperata* from India (matriline A4) by 9.2%, *F. kudremukhensis* and *F. nilagirica* (matriline A5) from India by 9.0%, *F. greenii* and *F. kirtisinghei* from Sri Lanka (matriline A6) by 9.7% and 9.8%, respectively, and *F. rufescens* from matriline A7 by 11.3%. High genetic diversity between the Chiang Mai specimens and the other South Asian matrilines suggest it could be a new species, which we describe below.

Fejervarya chiangmaiensis sp. nov. (Figure 4,5)

Holotype. Adult male (KIZ024057), from Ban Monjong, Omkoi District, Chiang Mai Province, Thailand (N17°28'16.93", E98°27'28.26", 460 m a.s.l.), collected by Chatmongkon Suwannapoom on 30 June, 2013.

Paratypes. Eleven males KIZ024053–56, KIZ024058, KIZ024126, KIZ024096–100; collected by Chatmongkon Suwannapoom, Zhiyong Yuan, and Fang Yan; other data same as the holotype.

Diagnosis. The new species assigns to *Fejervarya* on the basis of its position in the matrilineal phylogeny (Figure 3) and

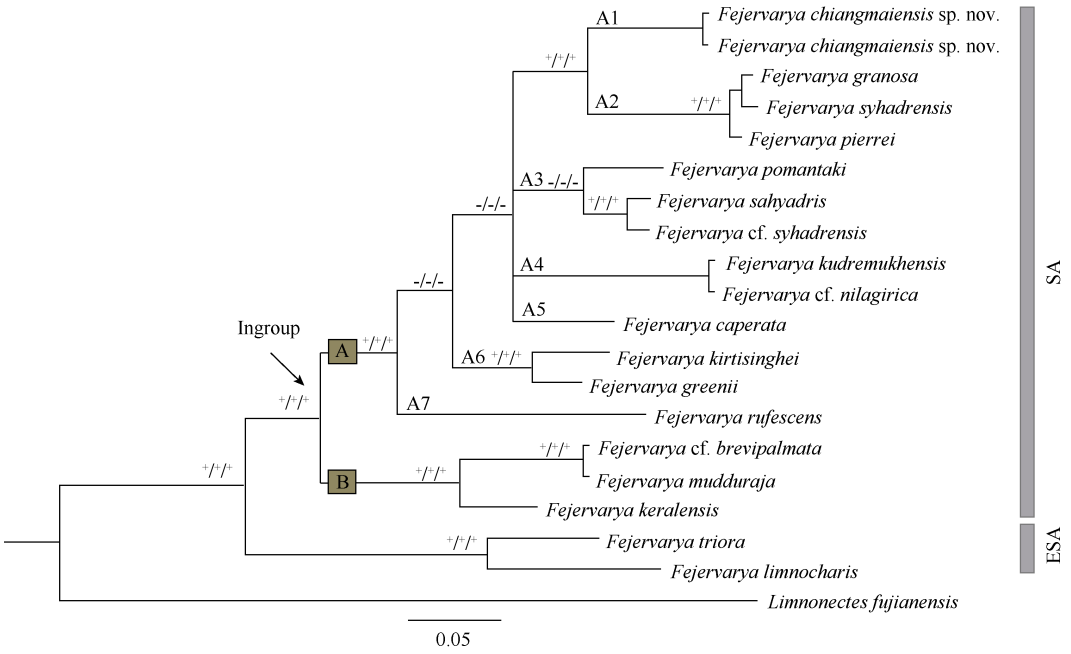

Figure 3 Matrilineal relationships among species of *Fejervarya* inferred from mtDNA 16S rRNA

Numbers above branches represent bootstrap support for Bayesian posterior probabilities (BPP; *>95%), maximum parsimony (MP; *≥75%), and maximum likelihood (ML; *≥75%), and "-" denotes low support (BPP<95% or BS<70%). Numbers near branches represent bootstrap support for Bayesian posterior probability MP and ML inferences, and (BPP/MP/ML). Scale bar represents 0.05 nucleotide substitutions per site. SA=South Asian group; ESA=East and Southeast Asian group.

Table 2 Matrix of uncorrected K2P distances among partial 16S rRNA gene sequences of members of *Fejervarya* (GenBank accession numbers follow species names)

Species	##	1	2	3	4	5	6	7	8	9	10	11	12	13
F. syhadrensis (AB488892)	1	-												
F. granosa (AB488895)	2	0. 6	-											
F. pierrei (AB488888)	3	0. 6	0. 4	-										
F. chiangmaiensis **sp. nov.** (KIZ024057)	4	6.7	6.4	6.0	-									
F. chiangmaiensis **sp. nov.** (KIZ024126)	5	6.7	6.4	6.0	0.0	-								
F. kudremukhensis (AB488898)	6	9.2	9.4	9.0	9.0	9.0	-							
F. nilagirica (AB167949)	7	9.2	9.4	9.0	9.0	9.0	0.0	-						
F. cf. *syhadrensis* (AB488893)	8	8.8	9.0	8.6	8.8	8.8	8.5	8.5	-					
F. sahyadris (AB530605)	9	9.0	9.3	8.8	9.0	9.0	8.1	8.1	1.2	-				
F. caperata (AB488894)	10	9.2	9.4	9.5	9.2	9.2	8.3	8.3	6.2	6.5	-			
F. greenii (AB488891)	11	10.0	10.3	10.3	9.7	9.7	9.7	9.7	8.3	8.6	7.6	-		
F. kirtisinghei (AY014380)	12	9.7	10.0	10.0	9.8	9.8	9.7	9.7	8.1	8.6	7.6	4.0	-	
F. rufescens (AB167945)	13	12.6	13.3	12.8	11.3	11.3	11.4	11.4	11.6	11.1	10.6	9.9	11.1	-
F. gomantaki (KR78086)	14	10.3	10.1	9.5	9.3	9.3	10.9	10.9	4.6	4.6	8.5	11.1	11.1	14.8

the following morphological characteristics: (1) slightly pointed snout; (2) comparatively poorly developed foot webbing; (3) lateral line system in adult absent; (4) characteristic "*Fejervarya*"-lines present; (5) femoral glands absent; (6) tympanum comparatively small; and (7) tibia length slightly more than a half of SVL. The new species is characterized by a combination of the following morphological characteristics: (1) small-size (males mean SVL 26.3-29.1 mm; *n*=12) (Table 3); (2) head length greater than head width; (3) tympanum small, discernible but unclear; (4) slightly elongated cylindrical internal metatarsal tubercle; (5) relative finger length (from longest to shortest) when addressed: II<IV<I<III; (6) webbing formula on foot=I 1-2 II

Figure 4 Dorsal (A) and ventral views (B) (scale bar, 10 mm); Ventral views of the right hand (C) and foot (D) (scale bar, 2 mm) of the male holotype of *Fejervarya chiangmaiensis* sp. nov. (KIZ024057) after preservation

Figure 5 Life photo of *Fejervarya chiangmaiensis* sp. nov. in *situ*; calling adult male paratype KIZ024056 from type locality

1-2½ III 2-3 IV 3-1 V; (7) cream vertebral line usually present medially lasting from between the eyes to the vent; males with paired dark vocal sacs (Figure 4); (8) dorsal and lateral parts of head and body, including body flanks, shagreened; posterior part of dorsum with distinct, elongate, glandular warts, continuing on dorsal surface of legs and arms; (9) dorsal skin showing rare, small, longitudinal folds arranged in series; and (10) advertisement call consisting of a long series of partially pulsed notes, each of which lasts 3.0±0.4 s, with 9-12 pulses/call (average 11.2±1.8), note interval of 1.8±0.6 s, and dominant frequency of 2.0 kHz.

Table 3 Measurements of type specimens (mm) (*n*=12 in males) of *Fejervarya chiangmaiensis* sp. nov.

Voucher number	**KIZ (Holotype) 024057**	KIZ 024054	KIZ 024097	KIZ 024098	KIZ 024126	KIZ 024100
Sex	male	male	male	male	male	male
SVL	26.3	29.1	28.8	26.9	27.9	29.1
HL	11.3	11.9	12.1	10.8	12.0	11.9
S-NL	2.2	2.5	2.6	2.3	2.5	2.6
N-EL	2.5	2.9	2.6	2.6	2.7	2.7
SL	4.8	4.7	5.0	4.7	5.2	5.3
EL	3.8	3.8	3.8	3.5	4.3	3.6
T-ED	2.0	2.3	2.8	2.0	2.6	2.6
HW	10.1	11.2	11.0	10.0	10.2	10.6
IND	2.5	2.7	2.9	2.5	2.8	2.7
IOD	1.9	2.1	1.5	1.9	1.7	1.7
UEW	3.4	3.4	4.0	3.5	3.6	3.2
FLL	18.0	19.8	19.1	16.6	18.5	18.0
LAL	11.6	12.1	11.0	10.6	12.1	11.6
FFL	5.0	5.2	6.0	4.9	4.9	5.3
HLL	47.7	51.2	50.0	47.2	48.8	49.9
TL	14.7	14.9	15.0	14.3	14.9	15.7
FL	14.4	15.7	15.8	14.3	15.7	15.7
IMTL	1.5	1.6	1.6	1.4	1.5	1.5
I-IV FL	II–-IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III
I-V TOEL	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV

Continued

Voucher number	KIZ 024055	KIZ 024058	KIZ 024099	KIZ 024096	KIZ 024053	KIZ 024056
Sex	male	male	male	male	male	male
SVL	28.2	26.9	28.2	29.1	29.1	27.6
HL	11.0	10.3	11.6	11.1	11.9	11.9
S-NL	2.2	2.3	2.4	2.5	2.7	2.5
N-EL	2.6	2.7	2.6	3.0	3.0	2.9
SL	4.8	5.0	5.0	5.4	5.7	5.4
EL	3.4	3.4	3.6	4.1	4.1	3.8
T-ED	2.0	2.7	2.6	2.3	2.7	2.2
HW	9.9	9.3	9.9	10.4	10.9	10.4
IND	2.2	2.2	2.5	2.8	2.9	2.6
IOD	1.8	1.9	1.7	2.1	2.4	2.1
UEW	3.2	3.5	3.6	3.7	3.9	3.3
FLL	17.3	17.2	16.9	18.6	20.6	17.2
LAL	11.8	11.5	12.0	12.4	12.6	11.3
FFL	5.1	4.7	5.3	6.0	5.6	4.6
HLL	47.2	46.8	45.8	51.5	50.9	47.6
TL	14.2	14.8	12.4	16.0	15.4	14.7
FL	15.6	14.4	15.5	15.8	16.2	14.1
IMTL	1.5	1.4	1.5	1.7	1.7	1.4
I-IV FL	II–IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III	II–IV–I–III
I-V TOEL	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV	I–II–V–III–IV

Abbreviations are listed in Material and Methods. KIZ=Kunming Institute of Zoology.

Description of holotype. (all measurements in mm; see Table 3): Adult male (KIZ024057; Figure 4). Small-sized frog specimen with SVL=26.3 mm, body habitus moderately stout (Figure 4A).

Head. Head of moderate size, head longer (HL 11.3 mm) than wide (HW 10.1 mm; HW/HL ratio 0.9), convex (Figure 4A). Snout more or less pointed from above; length (SL 4.8 mm) longer than horizontal diameter of eye (EL 3.8 mm) and interorbital distance (IOD 1.9 mm). Snout relatively distinct, protuberant; loreal region concave, angle to upper surface of snout rather vertical; canthus rostralis not sharp, rounded. Interorbital space slightly convex, much narrower (IOD 1.9 mm) than upper eyelid (UEW 3.4 mm) and narrower than internarial distance (IND 2.5 mm). Nostrils rounded, with a distinct flap of skin laterally, nostril slightly closer to snout than to eye (S-NL: 2.2 mm; N-EL: 2.5). Eyes comparatively small, protuberant; EL 33.5% of HL; upper eyelid with minute granules, pupil horizontal. Tympanum (TYD 1.4 mm) visible, but poorly distinct, rounded; slightly more than the half of eye diameter (TD 53.0% of ED), tympanum-eye distance (TYE 2.0 mm) half its diameter. Pineal ocellus indistinct. Choanae triangular, rounded; vomerine ridge absent, vomerine teeth in two oblique lines between choanae, beginning at anterior border of choanae, slightly extending beyond its posterior border. Tongue rather large, cordate, notably forked with two projections at tip; median lingual process absent; tooth like projections on maxilla absent.

Forelimbs. Lower arm short, rather strong (LAL 11.6 mm), 64.3% of forelimb length (FLL 18 mm). Fingers short, thin, without dermal fringe; webbing absent, finger tips bluntly rounded and not enlarged to disks (Figure 4C). Relative finger lengths from shortest to longest: II<IV<I<III (I: 5.5 mm; II: 5.1 mm; III: 6.6 mm; IV: 5.5 mm). Subarticular tubercles prominent, rounded, single, all present; a single palmar (thenal) tubercle large, well-developed, rounded. Prepollex oval, distinct; supernumerary tubercles absent.

Hindlimbs. Hindlimbs comparatively long, HLL (47.7 mm), about 1.8 times that of SVL (26.3 mm). Tibia (TL 14.7 mm) slightly longer than femur and subequal to foot length (FL 14.4 mm). Toes long, thin, toe tips blunt, slightly rounded, not enlarged to disks (Figure 4D). Relative toe lengths from shortest to longest: I<II<V<III<IV (I: 5.90 mm; II: 7.46 mm; III: 10.71 mm; IV: 13.5 mm; V: 9.5 mm). Subarticular tubercles prominent, elongated oval-shaped, protuberant, simple, all present. Inner metatarsal tubercle prominent, long and slightly compressed laterally (IMT: 1.5), 4 times the length of toe I. Foot webbing small, webbing formula I 1-2 II 1-2½ III 2-3 IV 3-1 V (Figure 4C,D). Dermal fringe along toe V absent. Inner tarsal ridge present, flat. Outer metatarsal tubercle present, prominent, elongated; supernumerary tubercles absent; tarsal tubercle absent.

Skin and skin glands. Snout smooth with rare indistinct dermal granules, nares with low dermal flaps, small tubercles on upper eyelid. Dorsal and lateral surfaces of head and body, including body flanks, shagreened, posterior part of dorsum with distinct, round glandular warts, continuing on dorsal surfaces of legs and arms; Dorsal skin showing rare, small,

longitudinal dermal ridges arranged in series. Anterodorsal part of thigh, anal region, dorsal surface of tibia, and tarsus with small granules; lateral sides of body, ventral surfaces of body and limbs smooth. Lateral-dorsal folds absent; lateral line system absent; "*Fejervarya*"-line present; supra-tympanic fold distinct, running from the posterior corner of eye backwards and downwards towards the incursion of the forelimb. Discernable macroglands, in particular rictal gland, absent.

Male secondary sexual characters. White nuptial pas present. Vocal sacs present, unique subgular pouch appear subdivided when inflated (Figure 5); pair of rounded openings in posterior part of mouth floor. No other discernable male secondary characters.

Coloration of adult in life. Dorsal ground color varies from dark green to dark brown; lateral sides greyish, ventral surfaces of body, head, and limbs whitish-cream. Marginal sides of gular area dark-greyish to blackish forming an M-shaped band (when inflated, vocal sac looks blackish; Figure 5). Transverse blackish-green to dark brown bands present on the dorsal surfaces of the thigh, tibia, and tarsus (Figure 5). Dorsal surfaces of fingers and toes greyish with indistinct transverse bands. Three to four dark brownish irregular blotches on each side of the upper jaw. Lateral sides of body with irregular grey-greenish and whitish blotches; dark greenish blotches get bigger towards the axilla and groin. Thin orange-yellowish mid-dorsal vertebral stripe runs from the anterior part of the interorbital space to the vent. Numerous fused irregular spots on the posterior surface of thigh dark green with thin dark brown reticulations in-between. Tympanum unclear dark green with darker circle in center, lower part of tympanum greyish. Iris greenish-bronze with dark reticulation; pupil horizontal.

Coloration in preservative. In alcohol the pattern described above was not obviously changed, although was slightly faded (Figure 4A). The yellowish tint faded the most, with greenish colors on the dorsal surfaces appearing brownish or gray-brownish (Figure 4A); the ventral sides look much lighter than in life (Figure 4B); the belly appeared whitish. Dorsum turned greyish brown with many large dark-gray to black spots, thin mid-dorsal stripe turned whitish, running from the interorbital space to the vent. Lateral with many small blackish dots, ventral immaculate except for a dark-grey M-shaped band across the gular area (Figure 4B). Transverse black bands on the dorsal surfaces of thigh, tibia, and tarsus were easily discernable.

Variation. Variation in meristic and morphometric characters among the type series are shown in Table 3. Individuals of the type series are generally similar in appearance, but show certain variation in coloration and dorsal pattern. Among the studied types, we found variation in the degree of development of mid-dorsal vertebral light line. Paratypes resemble the holotype in all aspects of morphology except for KIZ024053, KIZ024054 and KIZ024100, which do not have the thin whitish mid-dorsal stripe from tip of between eyes to vent (27%). In KIZ024098, the mid-dorsal line is broken in the scapular area. Variation in dorsal coloration is also observed: the head might be light dark green to dark brown and the dorsum is greyish brown with many large black spots.

Advertisement call. Calls were recorded at an air temperature of 26.0ºC. This species had both short and long calls, though the latter were not always emitted (Figure 2). The shorter call consisted of a series of pulsed notes. Each of these notes lasted 3.0±0.4 s and was composed of 9-12 pulses/call (average 11.2±1.8). The note interval was 1.81±0.598 s, the dominant frequency was 2.0±0.03 kHz, and the second harmonic was about 3 857±0.036 kHz. The call had a slight frequency modulation.

Etymology. The specific epithet *chiangmaiensis* is a Latinized adjective derived from the name of Chiang Mai Province, Thailand. We suggest the common English name "*Chiang Mai Rain-Pool Frog*" and vernacular name in Thai "*Kob-Nonglek Chiang Mai*", taken from "*Kob*" for frog, "*Nonglek*" for small swamp, "*Chiang Mai*" for Chiang Mai Province, Thailand.

Distribution and habitat. *Fejervarya chiangmaiensis* **sp. nov.** is, to date, known only from a single locality, encompassing a lowland farm in Ban Monjong, Omkoi District, Chiang Mai, northern Thailand (N17°28'16.93", E98°27'28.26"; 460 m a.s.l.). The frogs were found calling on clods of dirt in rice fields at night during rainfall; it appears that the species inhabits disturbed habitats, including agricultural areas. Females of the new species remain unknown. The new species was found in sympatry with *F. limnocharis*, *Occidozyga lima*, and *Hoplobatrachus rugulosus*.

Comparisons with other congeners.

Fejervarya chiangmaiensis **sp. nov.** can be distinguished from large- and medium-sized members of *Fejervarya* in external morphology, coloration, and acoustics. *Fejervarya chiangmaiensis* **sp. nov.** can be distinguished from members of the sister matriline, comprised of *F. granosa* (central Western Ghats, India), *F. pierrei* (Nepal, Bangladesh and E India), and *F. syhadrensis* (India, Pakistan, Bangladesh, Sri Lanka, and Nepal). The SVL of male *F. chiangmaiensis* **sp. nov.** (26.3-29.1 mm) overlaps with male *F. granosa* (29.1 mm; Kuramoto et al., 2007), *F. pierrei* (24.7-41.2 mm; Dubois, 1975), and *F. syhadrensis* (22-36 mm; Howlader, 2011b). However, the new species clearly differs in the dominant frequency of its advertisement call, which is higher (2.0 kHz) than that in *F. granosa* (1.7 kHz), but much lower than that in *F. pierrei* and *F. syhadrensis* (4.2 kHz and 2.7-4.1 kHz) (Kuramoto et al., 2007; Purkayastha & Matsui, 2012). Moreover, the new species can be further distinguished from *F. granosa* and *F. pierrei* by dorsum shagreened with rare low dorsal ridges and distinct glandular warts in the posterior part of the dorsum, and by moderately stout body habitus (vs. dermal ridges on the back well-pronounced, generally short or rounded and body shape relatively thick in *F. granosa*, Kuramoto et al., 2007; and vs. dorsum highly tuberculated, habitus stocky in *F. pierrei*, Dubois, 1975). The new species can be further distinguished from *F. pierrei* by relative finger lengths, with the second finger being shorter than the fourth finger (II<IV<I<III in *F. chiangmaiensis* **sp. nov.** vs. II=IV<I<III in *F. pierrei*; Howlader, 2011b). *Fejervarya chiangmaiensis* **sp. nov.** can be further differentiated from *F. syhadrensis* by head width less than head length, HW/HL rate 0.9 (vs. head broader than long, HW/HL rate 1.0 in *F. syhadrensis*, see Kuramoto et al., 2007) and by

relative finger lengths (II<IV<I<III in *F. chiangmaiensis* **sp. nov.** vs. I=II<IV<III in *F. syhadrensis*; Howlader, 2011b).

The body size of male *F. chiangmaiensis* **sp. nov.** differs from that of other species of *Fejervarya*. The new species (males 26.3-29.1 mm; Thailand) is smaller than *F. sengupti* (males 33.4-35.7 mm; Maghalaya, India; Purkayastha & Matsui, 2012) and much smaller than *F. kudremukhensis* (males 40.8-43.3 mm; Western Ghats, India; Kuramoto et al., 2007). The new species also clearly differs by the dominant frequency of the advertisement call, which is lower than that in both *F. sengupti* and *F. kudremukhensis* (2.0 kHz vs. 3.3 kHz and 3.6 kHz, respectively; as reported by Kuramoto et al., 2007; Purkystha & Matsui, 2012). *Fejervarya chiangmaiensis* **sp. nov.** can be further diagnosed from *F. sengupti* and *F. kudremukhensis* by dorsum shagreened with granules in the posterior part, and by head width less than head length, HW/HL rate 0.89 (vs. dorsum densely granulated with dermal transversal folds, and head notably wider than long, HW/HL rate 1.2 in *F. sengupti*, see Purkystha & Matsui 2012; and vs. dorsum with a few short dermal ridges and interrupted reversed V-shaped ridge in the scapular area, and head wider than long, HW/HL rate 1.1 in *F. kudremukhensis*, data for males, see Kuramoto et al., 2007).

Although comparative data are limited, the SVL of *F. chiangmaiensis* **sp. nov.** overlaps with the SVL of male *F. nepalensis* (23.0-37.8 mm, Nepal, NE India, Bhutan, Bangladesh; see Dubois, 1975). However, the new species clearly differs from the latter by relative finger length: II<IV<I<III and shagreened dorsum vs. II<I<IV<III and warted dorsum in *F. nepalensis*.

The following species of *Fejervarya* have greater male SVL values than that of *F. chiangmaiensis* sp. nov: *F. mysorensis* from India (37.0 mm: Dutta, 1997 as *Limnonectes*), *F. teraiensis* from Nepal (40.1-50.5 mm; Matsui et al., 2007), and *F. murthii* (35.0 mm: Dutta, 1997 as *Limnonectes*) and *F. nilagirica* (34.7-42.2 mm), two endemic species from India.

Although *F. asmati* from Bangladesh, *F. keralensis* from India, and *F. brevipalmata* from India overlap with the new species in body size (SVL 29.1-33.4 mm: Howlader, 2011a; 21.2-47.0 mm: Dutta, 1997, and 28.3-59.8 mm: Dutta, 1997 as *Limnonectes*, respectively), the presence of these species in northern Thailand appears to be highly improbable (see Bauer et al., 1995; Boulenger, 1905; Choudhury et al., 2001; Howlader, 2011b; Peters, 1871; Purkayastha & Matsui, 2012). The new species can be further differentiated from *F. asmati* by snout pointed in lateral view (vs. snout almost rounded in lateral view), by skin on dorsum shagreened without dermal folds in shape of inversed V (vs. skin on dorsum with transverse elongated ridges forming inversed V-pattern), by coloration of vocal sacs in breeding males forming a dark M-shaped pattern on the gular area (vs. characteristic butterfly-shaped spot on throat in males), and by the dominant frequency of the advertisement call, which is lower than that in *F. asmati* (2.0 kHz vs. 4.1-5.1 kHz) (Howlader, 2011b). The new species can be further distinguished from *F. keralensis* by having a comparatively larger head (HL/SVL 0.4 in *F. chiangmaiensis* sp. nov vs. 0.4 in *F. keralensis* males) and larger eyes (EL/SVL 0.1 in *F. chiangmaiensis* sp. nov vs. 0.1 in *F. keralensis*, data for males, see Kuramoto et al., 2007) and less developed small webbing; web formula: I 1-2 II 1-2½ III 2-3 IV 3-1 V (vs. wide almost complete webbing in *F. keralensis*; web formula: I 1-2 II 2-1 III 1-1 IV 1-1 V, Kuramoto et al., 2007).

The three other species of *Fejervarya* from the Western Ghats in southern India, *F. nilagirica*, *F. caperata*, and *F. mudduraja*, can be also differentiated from the new species on the basis of body size and proportions and by presence of warts and dermal ridges on the dorsum (Kuramoto et al., 2007). *Fejervarya nilagirica* is a large-bodied species and can be easily distinguished from the small-bodied *F. chiangmaiensis* **sp. nov.** (male SVL 26.3-29.1); and further distinguished by the numerous warts and dermal ridges on the dorsum (vs. smooth to shagreened dorsum with glandular warts in posterior part in *F. chiangmaiensis* **sp. nov.**) and by relatively smaller eyes, EL/SVL min 0.1-0.2 in the new species. Although *F. caperata* overlaps with *F. chiangmaiensis* **sp. nov.** in SVL (mean SVL being 33 mm in females and 29 mm in males), it can be distinguished by its relatively slender body habitus (vs. moderately stout body habitus in the new species), by long dermal ridges on the dorsum forming four longitudinal lines (vs. smooth to shagreened dorsum with glandular warts in posterior part in *F. chiangmaiensis* **sp. nov.**), and by relative finger lengths IV<II<I<III (vs. relative finger lengths II<IV<I<III in the new species). *Fejervarya mudduraja* can be distinguished by its large body size, with mean SVL of females being 45 mm (no information on male SVL available) (vs. small body size in *F. chiangmaiensis* **sp. nov.**, SVL 26.3-29.1), presence of long dermal ridges on the back arranged into four longitudinal lines (vs. smooth to shagreened dorsum with glandular warts in posterior part in *M. chiangmaiensis* **sp. nov.**), and by head wider than long, HW/HL=1.1 (vs. head width less than head length, HW/HL=0.9 in *F. chiangmaiensis* **sp. nov.**).

Fejervarya murthii (Tamil Nadu, Karnataka, south India) can be distinguished by the presence of two triangular patches bearing pearl-like papillae on the breast in males, and presence of the papillae in the anterior part of the lower jaw (Pillai, 1979) (vs. no such discernible papillae in *F. chiangmaiensis* **sp. nov.**).

The new species can be easily diagnosed from *F. teraiensis* inhabiting Nepal and NE India by its smaller body size (SVL 26.3-29.1 vs. SVL 37.8-44.1 in males of *F. teraiensis*; Howlader, 2011b), relative finger lengths (II<IV<I<III in *F. chiangmaiensis* **sp. nov.** vs. II=IV<I<III in *F. teraiensis*; Howlader, 2011b) and head width less than head length, HW/HL rate 0.9 (vs. head width almost equal to head length, HW/HL rate 1.0 in *F. teraiensis*).

Fejervarya rufescens from southern India can be distinguished by having stocky body habitus (vs. moderately stout habitus in *F. chiangmaiensis* **sp. nov.**), highly tuberculated skin on the dorsum with pronounced transverse dermal ridges forming an inverse V-pattern (vs. shagreened dorsum with rare low dorsal ridges in in *F. chiangmaiensis* **sp. nov.**), and reddish coloration on the dorsum in breeding males (vs. grayish or greenish dorsal coloration in breeding males of *F. chiangmaiensis* **sp. nov.**). Sri Lankan *F. kirtisinghei* and *F. greenii*, can be easily differentiated by the dorsum covered with well-developed long continuous dermal ridges (vs. shagreened

dorsum with rare low dorsal ridges, never forming continuous rows in *F. chiangmaiensis* **sp. nov.**).

Comparison of *F. chiangmaiensis* **sp. nov.** with certain dicroglossid species reported for the region is complicated due to their unclear taxonomic status. Locality of *F. brevipalmata*, originally designated as "Pegu, Myanmar", appears to be uncertain. This species likely occurs in the Western Ghats of southern India. Its taxonomic status is unclear (AmphibiaWeb, 2016; Boulenger, 1920). Furthermore, *F. sauriceps* and *F. parambikulamana*, two endemic species from Kerala and Karnataka of southern India, are known only from holotypes that appear to be lost. *Fejervarya sauriceps* is supposed to differ from other known *Fejervarya* by a very small tongue, unique triangular pit on the snout, a brown venter, and wide interorbital width (more than twice the upper eyelid width), whereas *F. parambikulamana* is diagnosed by smooth dorsum and comparatively longer legs (Kuramoto et al., 2007); both differ from *F. chiangmaiensis* **sp. nov.** Taxonomic validity and systematic status of these species requires further investigation (AmphibiaWeb, 2016; Frost, 2016) and some researchers doubt their validity (Matsui et al., 2007; Purkayastha & Matsui, 2012). Similar concerns have been raised on some other dicroglossid frog species known in regions nearby, in particular *F. altilabris* (Blyth) from Myanmar, *F. frithii* (Theobald) from Bangladesh, and *F. assimilis* (Blyth) and *F. brama* (Lesson) from India (Matsui et al., 2007). *Fejervarya chiangmaiensis* **sp. nov.** can be further differentiated by its smaller body size from *F. orissaensis* (Orissa, India; Dutta, 1997) (males 26.3-29.1 mm vs. 36.2-47.2 mm in *F. orissaensis* Dutta (1997) as *Limnonectes*). Furthermore, *F. chiangmaiensis* **sp. nov.** can be distinguished from the two other miniaturized species of *Fejervarya* (*F. sahyadris and F. chilapata*) by a larger SVL in males (male of *F. chiangmaiensis* **sp. nov.**, mean SVL=28.1±1.0; *n*=12 vs. males of *F. sahyadris and F. chilapata*, mean SVL=18.4±6.0 mm; *n*=10, SVL=20.0±7.0 mm; *n*=8, respectively), by lacking a white stripe on the upper lip (vs. white stripe present in *F. sahyadris and F. chilapata*), by lacking light dorsolateral lines (vs. light dorsolateral lines present in *F. sahyadris* and *F. chilapata*), by having shagreened dorsal and lateral surfaces of head and body including body flanks, posterior part of dorsum with distinct, round glandular warts, continuing on dorsal surfaces of legs and arms (vs. dorsal and lateral parts of head and body smooth; posterior part of back with indistinct, glandular warts in *F. sahyadris* and *F. chilapata*), and by different advertisement call (*F. sahyadris* (3.6-4.4 kHz) and *F. chilapata* (2.0 kHz) have a higher dominant frequencies than that of *F. chiangmaiensis* **sp. nov.** (2.0 kHz)).

Numerous differences in morphology, coloration, acoustics, and mtDNA gene sequences give support to recognizing the specimens collected in Chiang Mai Province of Thailand as a new species. Accordingly, description is necessary to accurately document the anuran biodiversity of Thailand.

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ZOOLOGICAL RESEARCH

Patterns of change in the population and spatial distribution of oriental white storks (*Ciconia boyciana*) wintering in Poyang Lake

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ABSTRACT

Using total counts in simultaneous annual surveys, we monitored the population size and spatial distribution of oriental white storks (*Ciconia boyciana*) wintering in Poyang Lake between 1998 and 2011. Results showed that Poyang Lake wetland is an important wintering ground for oriental white storks, with an annual average population number of 2 305±326. The population sizes in 2004, 2005, 2010, and 2011 were higher than the highest-ever estimate of its global population. In 2005, we recorded 3 789 individuals, which was the maximum population number within the period of 1998–2011. The storks inhabited 52 lakes, with the greatest distance between these lakes being 180.3 km. The storks presented a clustered distribution pattern in the Poyang Lake wetland, irrespective of the number of individuals or occurrence frequencies. Shahu, Dahuchi, Banghu, and Hanchihu were most frequently used lakes and had the largest annual average numbers of storks. There was a significant positive correlation between occurrence frequency and annual average number of storks in the lakes. Most of the lakes important for storks were covered by existing nature reserves, though some lakes outside the reserves were also frequently used. About 64.9%± 5.5% of the storks were found in nature reserves. In addition, the storks more frequently used and clumped in significantly larger flocks in lakes within nature reserves than lakes outside.

Keywords: Oriental white stork; Poyang Lake; Population size; Spatial distribution

INTRODUCTION

The oriental white stork (*Ciconia boyciana*) is a large migratory wader belonging to the genus *Ciconia*, family Ciconiidae, and order Ciconiiformes. It breeds primarily in the Amur and Ussuri basins along the border of Russia and China, and in northeastern China (BirdLife International, 2013; Liu & Li, 2008; Smirenski, 1991). Its main wintering grounds are in the lower Yangtze basin and southern China, including Taiwan and Hong Kong, with small numbers wintering in the Korean peninsula, Japan, the Philippines, northeastern India, Myanmar, and Bangladesh (BirdLife International, 2013). With a population size of only about 3 000 (Birdlife International, 2013), this species is listed as endangered on the IUCN Red List of Threatened Species, and is also included in Appendix I of the Convention on International Trade in Endangered Species (CITES). In China, it is also listed as a level I wild bird under special state protection (China Wildlife Propagation Institution for Protection, 1989).

The Poyang Lake wetland is an important wintering ground for the oriental white stork. Field surveys on its wintering population have been conducted by Chinese ornithologists since the 1980s, with the first report of 200 individuals observed in Poyang Lake presented in 1983 (Wu, 2002). This species and specific population has attracted increasing attention, especially after the establishment of the Poyang Lake Nature Reserve. Ji et al. (2006) observed 1 258 individuals by aerial survey across Poyang Lake in the winter of 2006, which accounted for 42.0% of its global population. In the winter of 2008, 3 909 individuals were observed in Poyang Lake, which was the highest-ever recorded global population (Wu et al.,

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2010). Wu et al. (2010) also noted more than 300 individuals – exceeding 10.0% of its global population – in each of 11 inner lakes of the Poyang Lake wetland.

Until now, few studies on oriental white storks covering the entire Poyang Lake wetland have been conducted due to the numerous inner lakes within the area. Currently, all available reports are confined to the Poyang Lake National Nature Reserve (PLNNR). In this study, we monitored the population size and distribution of oriental white storks across 77 inner lakes of the Poyang Lake wetland to better understand the population dynamics and identify important sites for the stork within the area.

MATERIALS AND METHODS

Study area

Poyang Lake (E115°49′–116°46′ and N28°11′–29°51′) is the largest freshwater lake in China, covering a drainage basin of 162 000 km^2. It is in the north of Jiangxi Province and on the south bank of the Yangtze River. It has a subtropical humid climate with a distinct seasonal shift, characterized by an average annual precipitation of 1 636.4 mm and a mean annual temperature of 17–17.8 °C. Poyang Lake receives runoff primarily from five tributaries, namely the Ganjiang, Xiuhe, Fuhe, Xinjiang, and Raohe rivers, and discharges into the Yangtze River in the north; however, reversed flow sometimes occurs due to an elevation of the Yangtze River water level. The Poyang Lake water level shows obvious seasonal fluctuation and can be divided into wet (April to September) and dry seasons (October to March) (Wang, 2004; Ye et al., 2011). In the wet season, the floodplains are inundated and form a large lake with a surface area of more than 3 000 km^2. In the dry season, the lake shrinks to less than 1 000 km^2 and forms a narrow meandering channel surrounded by numerous individual, dish-shaped inner lakes (Wang, 2004).

The Poyang Lake wetland is an internationally important wintering ground for migratory waterbirds. About 310 bird species, including 155 winter visitors and 107 summer visitors, inhabit Poyang Lake. It has been estimated that over 98.0% of the global population of Siberian cranes (*Grus leucogeranus*), 80% of oriental white storks, 50% of white-napped cranes (*G. vipio*), and 50% of swan geese (*Anser cygnoides*) winter in Poyang Lake (Wu, 2002). Poyang Lake is also an important wintering ground for the hooded crane (*G. monacha*), common crane (*G. grus*), tundra swan (*Cygnus columbianus*), and Eurasian spoonbill (*Platalea leucorodia*). Nowadays, there are three nature reserves dedicated to the protection of migratory waterbirds, namely PLNNR, Jiangxi Poyang Lake Nanjishan Wetland National Nature Reserve (NWNNR), and Duchang Provincial Nature Reserve for Migratory Waterbird Conservation (DPNR).

Bird census

We monitored the population size and distribution of oriental white storks across 77 lakes of the Poyang Lake wetland (Figure 1). The study area covered most of the inner lakes in all 13 counties of Poyang Lake, including Nanchang, Xinjian, and Jinxian – which are part of the Nanchang municipality – Gongqingcheng, Ruichang, Duchang, Xingzi, Hukou, Pengze, Jiujiang, and Lushan District – which are part of the Jiujiang municipality – and Yugan and Poyang in the Shangrao municipality.

Each winter, from 1998 to 2011, we used total counts to investigate the number of oriental white storks across the 77 lakes on the same day to avoid repeats or omissions due to the frequent movement of the storks. Surveys were conducted in December or January in most years, except for 2009 and 2010 when the surveys were conducted in February. Forty survey groups were organized each year; each group was comprised of one or two professionals and one guide, equipped with monoculars (25–75×82) and binoculars (10×56). The survey members came from the Administration of PLNNR, NWNNR, and DPNR, Jiangxi Normal University, Jiangxi Agricultural University, Jiangxi Academy of Forestry, and local volunteers. Each year, investigators undertook a two-day training program on waterbird survey methods before the formal field survey. Each group began its land survey in the morning. If some sites were not reachable by foot, a boat was used. Whenever the investigators encountered oriental white storks, they stopped to observe, count, and record the number.

Data analysis

To investigate whether significant changes in population numbers had occurred in Poyang Lake, we chose the population size of oriental white storks as the dependent variable and time (year) as the independent variable. The occurrence frequency and average number of storks in each lake were used to evaluate the importance of lakes to the storks. We also interpolated a raster surface from lake importance using an inverse distance weighted (IDW) technique to illustrate stork distribution (Tang & Yang, 2006). The generated raster size was 0.05°×0.05°. To compare the frequency of lake use under different protection statuses, we used a one-sample Kolmogorov-Smirnov test to examine whether the data were normally distributed. If the distribution was normal, analysis of variance (ANOVA) was implemented; if not, two independent sample tests were used to examine whether there was a significant difference in use intensity between protected and unprotected lakes.

RESULTS

Population size

Between 1998 and 2011, the average population size of oriental white storks wintering in Poyang Lake was 2 305±326. Population numbers did show considerable fluctuations, but no significant patterns of change (Figure 2). There were 2 832 storks in 1998, but only 838 in 2000. The population then showed a significant exponential increase between 2000 and 2005 (R^2=0.935, F=57.209, P=0.002), and reached its maximum number of 3 789. After 2005, the population number decreased linearly (R^2=0.939, F=46.407, P=0.006) to the minimum number of 430 in 2009. The population increased sharply to 3 445 in 2010, and remained at a similar size in 2011. The numbers of oriental white storks in Poyang Lake in winter 2004, 2005, 2010, and 2011 were higher than the highest-ever estimate (3 000) of the global population.

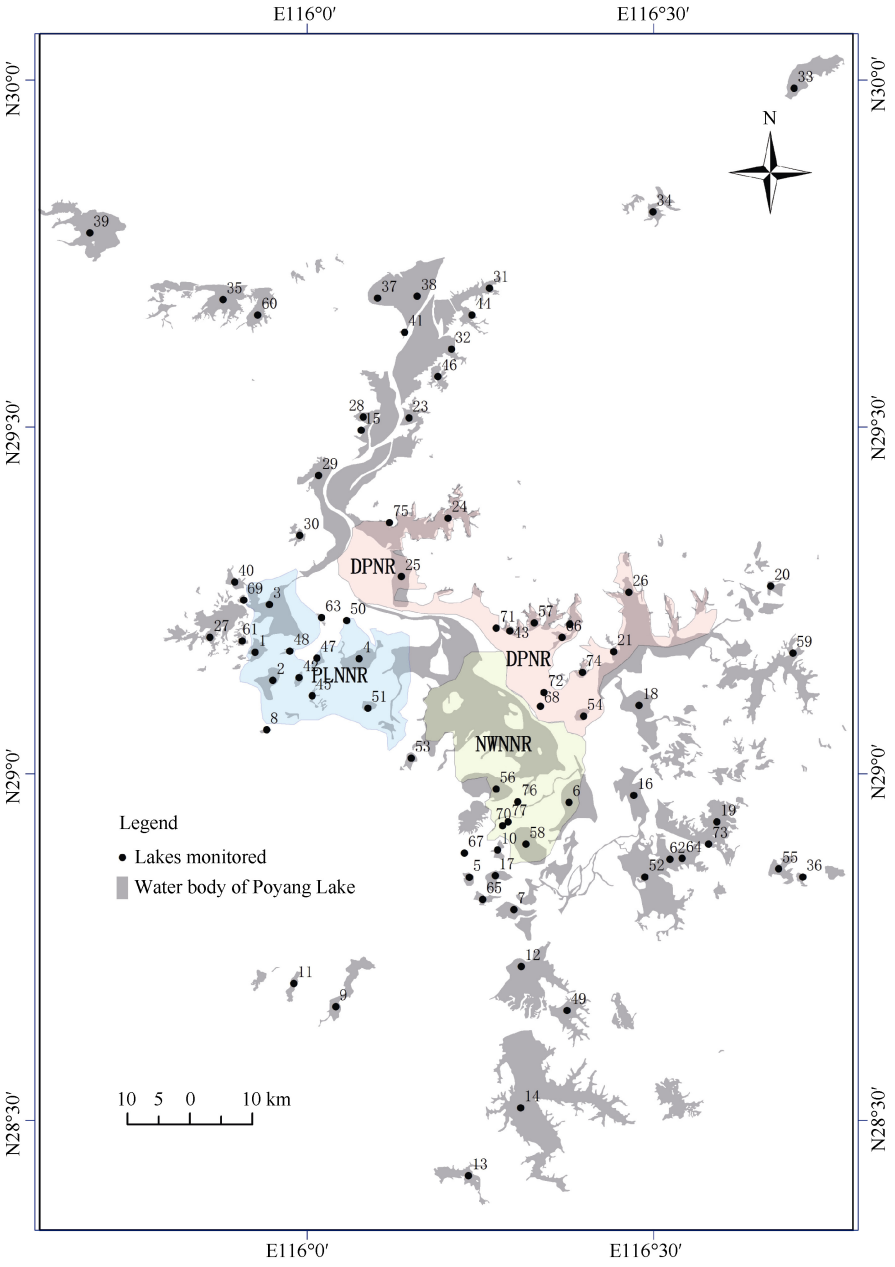

Figure 1 Map showing locations of the lakes involved in monitoring the wintering population of the oriental white stork in the Poyang Lake wetland

The highlighted polygons are nature reserves, including Poyang Lake National Nature Reserve (PLNNR), Nanjishan Wetland National Nature Reserve (NWNNR), and Duchang Provincial Nature Reserve (DPNR). Specific lakes are listed: 1. **Shahu**; 2. **Dahuchi**; 3. **Banghu**; 4. **Dachahu**; 5. **Xihu** (in Nanchang county); 6. **Donghu**; 7. **Chengjiachi**, 8. **Linghu**; 9. Yaohu; 10. **Sanhu**; 11. Aixihu; 12. Jinxihu; 13. Qinglanhu; 14. **Junshanhu**; 15. Zhulinhu; 16. **Nanjianghu**; 17. **Linchonghu**; 18. **Hanchihu**; 19. **Dalianzihu**; 20. **Qihu**; 21. Changxihu; 22. Huamiaohu; 23. Gaoqiaohu; 24. **Xinmiaohu**; 25. **Jishanhu**; 26. **Xihu** (in Duchang county); 27. **Nanhu**; 28. **Meixiihu**; 29. Shilihu; 30. **Liuhuachi**; 31. **Beiganghu**; 32. **Boyanghu**; 33. **Taibohu**; 34. **Fanghu**; 35. **Saihu**; 36. **Wanhu**; 37. Fanglanhu; 38. **Xieshanhu**; 39. **Chihu**; 40. **Sixiahu**; 41. **Gushanhu**; 42. **Changhuchi**; 43. Zhuantanghu; 44. **Nanganghu**; 45. **Xianghu**; 46. **Zaohu**; 47. **Zhonghuchi**; 48. **Zhushihu**; 49. **Chenjiahu**; 50. **Meixihu**; 51. **Candouhu**; 52. Chaqihu; 53. **Dawuhu**; 54. Duimianshan; 55. Zhangonghu; 56. Nihu; 57. Nanxihu; 58. **Sanniwan**; 59. **Zhuhu**; 60. Qilihu; 61.**Changhu**; 62. **Nanjianhu**; 63. **Canghu**; 64. **Luojiaohu**; 65. Nanhu (in Yugan county); 66. Shuhu; 67. **Yufeng**; 68. **Zhouxinihu**; 69. Zhoubianhu; 70. **Zhanbeihu**; 71. Huangjinzui; 72. Sishan; 73. Ganquanzhou; 74. Panhu; 75. Mayinghu; 76. **Beijiahu**; 77. **Changhu**.

* The boldfaced lakes indicate that oriental white storks were recorded on these lakes; the black spots represent lakes within Poyang Lake.

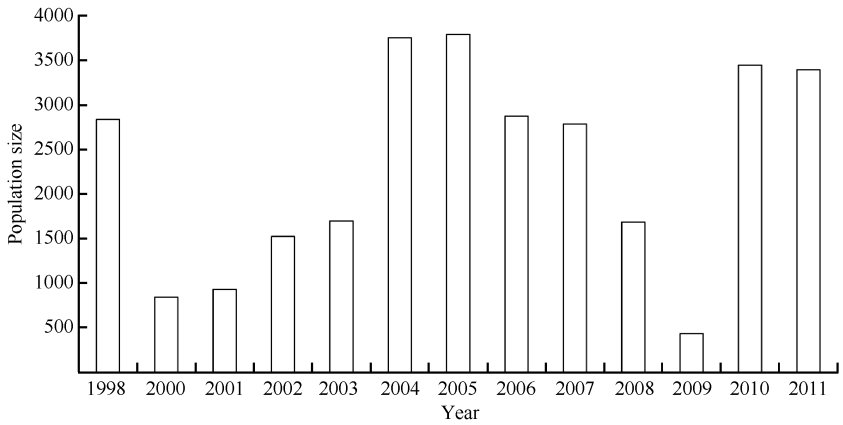

Figure 2 Dynamics of the oriental white stork population in Poyang Lake between 1998 and 2011

Spatial distribution

In the Poyang Lake wetland, 52 sub-lakes were used by wintering oriental white storks (Figure 1). The greatest distance between these lakes was 180.3 km. The numbers of oriental white storks in 32 sub-lakes exceeded 1% of the global population (Table 1). The maximum numbers of oriental white storks in each of the 32 lakes over the 14-year period, in descending order, were: Shahu, 1 770; Chengjiachi, 1 738; Dahuchi, 1 559; Nanhu, 1 510; Dachahu, 1 155; Banghu, 1 011; Zhonghuchi, 790; Sanhu, 686; Hanchihu, 674; Yufeng, 600; Candouhu, 566; Zhuhu, 565; Changhu, 531; Dalianzihu, 450; Xianghu, 328; Meixihu, 303; Linchonghu, 259; Liuhuachi, 210; Xieshanhu, 200; Sanniwan, 198; Qihu, 197; Dawuhu, 135; Zaohu, 121; Junshanhu, 114; Donghu, 92; Xihu (Nanchang) 91; Luojiaohu, 90; Beiganghu, 90; Zhushihu, 86; Nanshanhu, 85; Zhanbeihu, 80; Linghu, 62; Meixiihu, 56; Nanjianghu, 55; Changhuchi, 54; Chenjiahu, 37; Taipohu, 32; and Xihu (in Duchang county), 30. The average flock size of oriental white storks in these lakes was 286±34. The lakes where the number of oriental white storks exceeded 40.0% of the global population (i.e., 1 200) included Chengjiachi in Yugan county (1 738 individuals, winter 2004), Dahuchi in PLNNR (1 277, winter 1998; 1 559, winter 2011), and Shahu in PLNNR (1 770, winter 2007).

Table 1 Lakes with an oriental white stork population exceeding 1% of the global population

Lake	Year													Average
	1998	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	
Shahu							825	336	1770				960	973
Chengjiachi						1738								1738
Dahuchi	1277	294	261	43	325	440				368			1559	571
Dachahu		69					303	108	93			1155		346
Banghu			159				347	93	47	1011	203	843	64	346
Zhonghuchi	51		61	474		790	172	175						287
Sanhu												686		686
Hanchihu			94		450	132	674	617	338					384
Yufeng	600													600
Candouhu					566									566
Zhuhu							565	327	168	102		231		279
Changhu	34			531		51	59	91	139		129			148
Dalianzihu			78			450	419	428	45			55	41	217
Xianghu	328										31		321	227
Meixihu							303							303
Linchonghu							39	130				72	259	125
Liuhuachi		210												210
Xieshanhu												200		200

Continued

Lake	Year													Average
	1998	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	
Sanniwan				198										198
Qihu					132	66	30	197		135				112
Dawuhu								130	135					133
Zaohu	121													121
Junshanhu				114										114
Nanhu			88					100						94
Donghu	92													92
Xihu (in Jinxian county)	91													91
Beiganghu		90												90
Luojiaohu	90													90
Zhushihu	86													86
Nanshanhu		85											56	71
Zhanbeihu			36									80		58
Linghu				62										62
Meixiihu												56		56
Nanjianghu			46		55								30	44
Changhuchi													54	54
Chenjiahu								37						37
Taibohu				32										32
Xihu (in Duchang)								30						30

The lack of data in some years indicates that the numbers of oriental white storks in corresponding lakes were less than 1% of the global population number.

We determined that 12–23 lakes (17 on average) were used by oriental white storks each year. Storks were found more than eight times in 12 lakes in 13 surveys (Figure 3). Among these lakes, oriental white storks were found in Dahuchi 11 times in 13 surveys, with an annual average population of 418±157. However, the individual number in Dahuchi changed drastically. For example, only five storks were observed in 2006, but 1 559 were observed in 2011. The maximum average number of oriental white storks was found in Shahu (437±208). Taibohu, Fanghu, and Nanhu (in Gongqingcheng District) were used by the storks in nine years of the study period, but the average flock sizes were only 26±13, 15±3, and 11±2, respectively.

The storks showed a clustered distribution pattern at the inner lakes of Poyang Lake wetland (Figure 4A). In winter, the

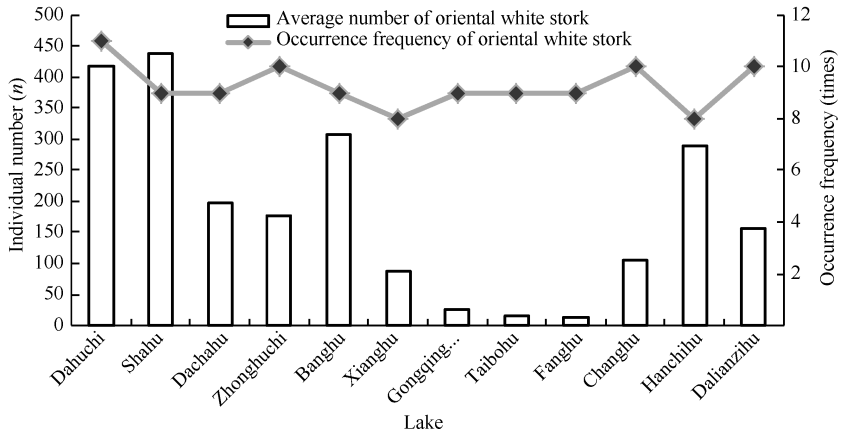

Figure 3 Occurrence frequencies and average numbers of wintering oriental white storks at lakes where storks were recorded more than six times between 1998 and 2011

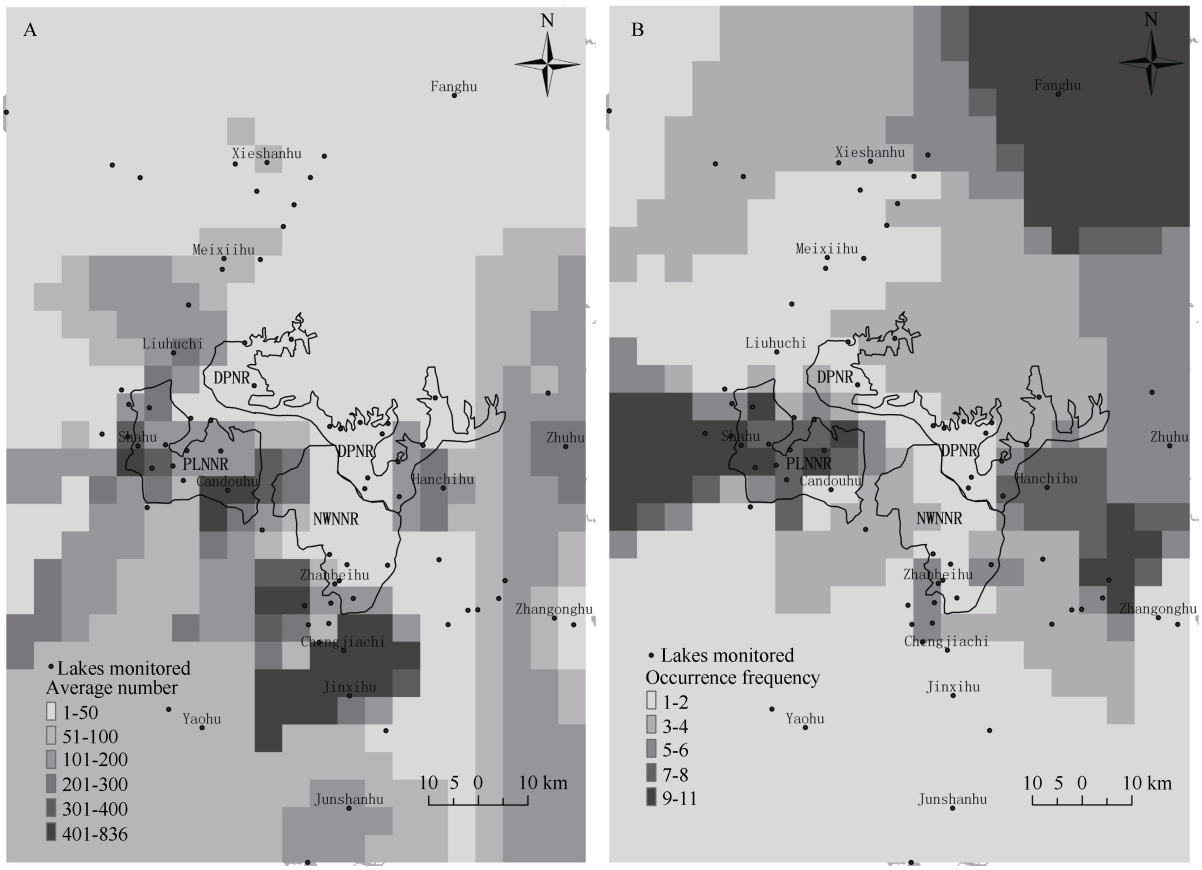

Figure 4 Distribution of wintering oriental white storks in Poyang Lake based on (A) average annual number and (B) occurrence frequencies

stork population was generally clumped on a few lakes, with a small proportion scattered across other lakes. A large proportion of the Poyang Lake population was observed on Shahu, Dahuchi, Banghu, and Hanchihu lakes, with average numbers of 418±157, 437±208, 308±123, and 290±95, respectively; large flock sizes were observed at the first three of these lakes. For example, in winter 2008, 60.2% of the oriental white stork population wintering in Poyang Lake was observed at Banghu. In every year of the study period, the largest flock sizes were found on just three lakes, where the total number of storks could be as high as 74.0%±3.6% of the overall population wintering in Poyang Lake. In winter 2008, the total number of oriental white storks on Dahuchi, Banghu, and Qihu accounted for 90.2% of the entire population at Poyang Lake. According to the occurrence frequencies of oriental white storks at these lakes, distribution across the lakes was uneven and clustered (Figure 4B). There was a significant positive correlation between occurrence frequency and average number of storks on the lakes (r=0.274, *P*=0.049, *n*=52).

In Poyang Lake, most lakes of importance to the storks were found within nature reserves. The average number of storks distributed in PLNNR, NWNNR, and DPNR was 1 428±217, accounting for 64.9%±5.5% of the total population wintering in Poyang Lake (Figure 5). Among these nature reserves, PLNNR possessed the highest number of storks, which corresponded to 55.7%±5.1% of the total population. The number of storks wintering in PLNNR increased significantly after 2007, and was normally more than 58.5% of the total population in subsequent years. In 2011, the number of storks in PLNNR was 87.6%. Accordingly, the number of storks outside the nature reserves tended to decrease after 2004 (Figure 5). In 2004 and 2006, 64.3% and 65.7% of the total Poyang Lake oriental white stork population, respectively, was found outside the nature reserves. However, the numbers decreased to 7.2% during 2007–2009, and then increased to 38.9% in 2010.

The storks used lakes within the nature reserves significantly more often (3.5±0.7, *n*=33) than those outside the nature reserves (2.5±0.4, *n*=44) (Mann-Whitney *U*=209.0, *P*=0.028). The average flock size within the nature reserves (84±26 individuals, *n*=33) was significantly larger than that outside the reserves (77±25 individuals, *n*=44) (Mann-Whitney *U*=159.0, *P*=0.002). Lakes within national nature reserves were used significantly more often (5.8±0.9 times) than those within the provincial nature reserves (0.8±0.3 times) (Mann-Whitney *U*=29.5, *P*=0.000) and those outside the nature reserves (2.4±0.4 times) (Mann-Whitney *U*=189.0, *P*=0.001). Moreover, average flock size on lakes within national nature reserves (153±40 individuals) was significantly larger than that within the provincial nature reserves (2±1 individuals) (Mann-Whitney *U*=17.5, *P*=0.000)

and that outside the nature reserves (77±18 individuals) (Mann-Whitney U=234.0, P=0.011). However, the storks inhabited lakes within the provincial nature reserves significantly less often than they inhabited lakes outside the nature reserves (Mann-Whitney U=209.0, P=0.028), and a significantly smaller flock size was found on lakes within the provincial nature reserves than on lakes outside the nature reserves (Mann-Whitney U=159.0, P=0.002).

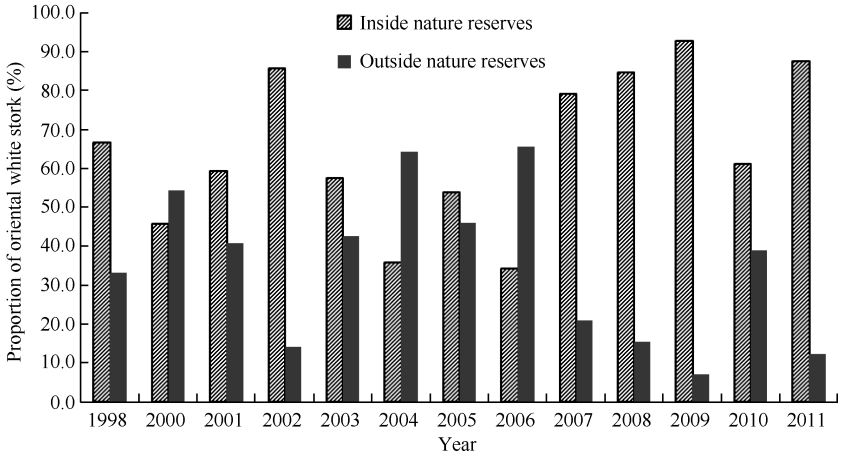

Figure 5 Distribution of wintering oriental white storks inside and outside nature reserves within Poyang Lake between 1998 and 2011

DISCUSSION

Population number

Oriental white storks mainly winter in the middle and lower reaches of the Yangtze River floodplain, with Poyang Lake possessing the largest wintering population in the world (Wu, 2002). Therefore, the fluctuations of stork populations in Poyang Lake are a good reflection of its global population dynamics. With only about 3 000 oriental white storks globally, this species is considered endangered (BirdLife International, 2013). We found that the number of storks in Poyang Lake in 2004, 2005, 2010, and 2011 exceeded the highest-ever estimated global population. The number in 2005 reached 3 789, and was approximately 3 400 in 2010 and 2011. This might be because of population increase or previous underestimation of population size. Zhu et al. (2012) observed 4 052 storks in December 2011, which was the largest recorded number of storks in Poyang Lake. The tendency for population growth in PLNNR between 1983 and 2011 also provides evidence toward an overall population increase. One reason for this increase might be the better protection of its habitats and decrease in illegal hunting when these habitats were enclosed in nature reserves over the last 20 years (An et al., 2007; Su et al., 2000). Another reason could be that many storks were forced to winter in Poyang Lake due to habitat deterioration of other wetlands in the middle and lower reaches of the Yangtze River floodplain (Cui et al., 2013; Liu et al., 2013). Although the population has increased obviously in recent years, it has shown extreme annual fluctuations. There were 2 832 individuals observed in 1998, but only 838 in 2008, accounting for 29.6% of the population size observed 10 years previously. The population then showed exponential growth after 2000, rising to 3 789 in 2005. However, this was again followed by a drastic reduction after 2006, and by 2009 only 430 individuals were found. In 2009, our field survey was carried out at the end of winter, when many storks may have left Poyang Lake and started their migration toward breeding grounds, thus resulting in an underestimation of the stork population for that year.

A satellite tracking study found that oriental white storks sometimes move hundreds of kilometers over wintering ground (Van den Bossche et al., 2001). They can move between Poyang Lake in Jiangxi Province and Shengjin Lake in Anhui Province, and use an extensive area in winter (Wu et al., 2001). In our study, the field survey was implemented within one day to ensure synchronization. Nevertheless, some storks may also disperse among sub-lakes of Poyang Lake or among lakes in the middle and lower reaches of the Yangtze River floodplain, which could result in a biased estimated population. Consequently, undertaking further synchronization surveys is necessary to accurately estimate the wintering population of the oriental white stork. In addition, independent surveys conducted by other organizations should be used as references. For example, regular monthly waterbird monitoring conducted by the PLNNR could help improve the accuracy of population estimates.

Another reason for the population fluctuation might also lie in the high mortality of oriental white storks during migration. Earlier studies found that *Ciconia ciconia*, a sibling species of oriental white stork, has a high rate of mortality (52%–74%) during migration in the first winter (Van den Bossche et al., 2002), and while adult mortality rates decrease, they can remain as high as 25%–50%. Satellite tracking has also revealed that the mortality rate of oriental white storks is higher in wintering grounds and during migration (Van den Bossche et al., 2001), with five out of six storks failing to reach adulthood and perishing at stopover or wintering grounds. In wintering areas, storks can be injured by ice forming at night, even in winters without extreme low temperatures (Yu, 2013). Thus,

high mortality during migration or on wintering grounds might result in the obvious fluctuations of the oriental white stork population. At the same time, factors such as deforestation, spring fire, reclamation of wetland, and overfishing also seriously threaten the survival of storks in breeding areas (BirdLife International, 2013). Therefore, it is not surprising that dramatic fluctuations in the oriental white stork population on Poyang Lake were observed within the study period.

Population distribution and *in-situ* conservation

Most of the important wintering lakes for oriental white storks were enclosed in nature reserves within Poyang Lake. Over the past 13 years, an average of 64.9%±5.5% of the Poyang Lake population has been observed within nature reserves. The storks were also commonly found clumped on only a few lakes. The highest occurrence frequencies and largest flock sizes were found on Shahu, Dahuchi, Banghu, and Hanchihu lakes located in PLNNR, indicating its critical importance for wintering storks. The average number of storks that wintered in PLNNR was 55.7%±5.1% of the total. In 2011, the proportion of storks in PLNNR rose to 87.6% of the total population. The clumped distribution pattern might result from less human disturbance and more suitable water depth in PLNNR. With the establishment of nature reserves such as NWNNR and DPNR in recent years, an increasing number of lakes of importance to the oriental white stork have been encompassed by nature reserves. Lakes such as Changhu, Zhanbeihu, and Sanniwan in NWNNR, and Xihu in DPNR, were observed with more than 1% of the global population of oriental white storks.

PLNNR, NWNNR, and DPNR are located adjacent to each other. All reserves have undergone rapid development over the past 10 years. They are well-resourced and fully equipped with convenient transportation, advanced communication, and professional staff. We found that storks occurred significantly more often and aggregated in larger flocks at lakes within PLNNR and NWNNR than lakes outside these reserves. This is likely a reflection of less human disturbance and wetland degradation and more suitable lakes for wintering storks. DPNR covers the main water body of Poyang Lake and has large areas of deep water, which lowers its suitability as wading bird habitat; therefore, storks within this area were smaller in flock size and at lower frequency than those found in other lakes.

The main human activity that potentially affects the oriental white stork in Poyang Lake is traditional fishery operation. This unique mode of fishing has been popular in Poyang Lake for many hundreds of years. Local fisherman build short dams to entrench dish-shaped lakes in autumn to reserve as many fish as possible; at the start of winter, fishermen dig drainage ditches to drain off lake water and install fish pots in the opening of the ditch to catch fish, which leads to a gradual reduction in the water level until the lake dries up (Guo et al., 2014). The available habitats for the oriental white stork thus change dramatically and the storks are forced to seek new suitable habitats (Liu et al., 2011). Traditionally, administrators of all nature reserves, from national to county level, have been unable to interfere in decisions regarding water management, which have been entirely governed by fishery production to secure maximum economic benefit. Nowadays, how to harmonize traditional fishery production with the conservation of large wading birds within Poyang Lake is an issue that requires urgent resolution. Therefore, we propose that a proper ecological compensation system be established to ease the conflict between waterbird conservation and utilization of fishery resources. Given the remarkable spread of oriental white storks across the wintering grounds of this lake wetland, we also suggest that a monitoring and protection network for oriental white storks be given high priority as part of the conservation of large wading birds in the middle and lower reaches of the Yangtze River of China.

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Molecular characterization and functional analysis of a piscidin gene in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

The piscidin family, which includes potent antimicrobial peptides with broad-spectrum activity, plays an important role in the innate immune system of fish. In this study, we cloned piscidin-5-like type 3 (*Lcpis5lt3*) in large yellow croaker (*Larimichthys crocea*). Multiple alignments with other known piscidins revealed amino acid conservation throughout the fish, especially at the signal peptide (22 amino acids). The phylogenetic tree confirmed that Lcpis5lt3 and large yellow croaker piscidin-5-like proteins were grouped together to form a branch. Quantitative real-time PCR revealed that *Lcpis5lt3* was expressed in a wide range of tissues, including the brain, muscle, gill, head kidney, intestine, kidney, liver, and spleen. The highest mRNA expression level of *Lcpis5lt3* was found in the spleen. After *Vibrio alginolyticus* infection, mRNA expression was rapidly upregulated in the liver, head kidney, gill, kidney, and intestine at 4, 8, 12, and 24 h post infection (hpi), whereas there were no significant changes in the spleen. The antimicrobial spectrum showed that the synthetic mature peptide of Lcpis5lt3 exhibited different activity *in vitro* against various bacteria, such as *Aeromonas hydrophila*, *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, *Staphylococcus aureus*, and *Listeria monocytogenes*. In addition, survival rates from the *in vivo* assay indicated that the synthetic peptide of Lcpis5lt3 increased the survival rate of large yellow croaker after *V. alginolyticus* challenge, resulting in a decline in bacterial burden and mRNA expression levels of interleukin-1β, interleukin-10, and tumor necrosis factor-α. These data suggest that Lcpis5lt3 plays an important role in innate immunity in large yellow croaker and might represent a potential therapeutic agent against pathogen invasion.

Keywords: Antimicrobial activity; Large yellow croaker; Piscidin; Survival rate; *Vibrio alginolyticus*

INTRODUCTION

Large yellow croaker (*Larimichthys crocea*) is an economically important marine species of cultured fish (Niu et al., 2013). However, the aquaculture of large yellow croaker has suffered significant economic losses due to various infectious diseases caused by marine microorganisms such as *Vibrio alginolyticus* (Chen et al., 2003; Liu et al., 2016). *Vibrio alginolyticus* is an important pathogen that can cause disease in marine cultured fish (Samad et al., 2014). Infection leads to the upregulation of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) (Kayansamruaj et al., 2014; Ringø, 2011; Seppola et al., 2008). Infection also increases bacterial burden, which triggers multiple inflammatory mechanisms. Therefore, bacterial burden is an important indicator for innate host immunity in response to infection (Gomes et al., 2013). Increased lethality is also observed in various teleosts infected with pathogens (Chen et al., 2014; Li et al., 2014a). Nowadays, a variety of effective vaccines and medicines have been developed to control marine pathogens; however, these drugs often negatively affect the marine environment and fish themselves (Cabello et al., 2013). Therefore, there is an increasing demand for effective and environmentally friendly commercial therapeutics against marine microorganisms. In this respect, considerable attention

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has been paid to antimicrobial polypeptides (AMPs), known as endogenous antibiotics (Mukherjee & Hooper, 2015).

AMPs, a family of peptides and proteins with low molecular weight, are present in virtually all life forms (from prokaryotes to eukaryotic plants and animals) (Zasloff, 2002). These peptides are critical components of the innate immune system in low vertebrate hosts (Corrales et al., 2010; Lauth et al., 2002). Many AMPs have been identified from fish, including cathelicidin, histone-derived peptides, defensin, and hepcidin (Katzenback, 2015). Piscidin family proteins possess antimicrobial activity and include pleurocidin, moronecidin, chrysophsin, and dicentracin (Masso-Silva & Diamond, 2014; Umasuthan et al., 2016). In teleosts, piscidin genes have been cloned and reported in some species, including Atlantic cod (*Gadus morhua*) (Fernandes et al., 2010; Ruangsri et al., 2012), rock bream (*Oplegnathus fasciatus*) (Umasuthan et al., 2016), tilapia (*Oreochromis niloticus*) (Lin et al., 2016; Peng et al., 2012), hybrid striped bass (Noga et al., 2009; Salger et al., 2011; Silphaduang & Noga, 2001), and mandarin fish (*Siniperca chuatsi*) (Sun et al., 2007). The piscidin gene shares a common prepropeptide structure consisting of a signal peptide, a mature peptide, and a C-terminal prodomain of varied sequence and length (Lauth et al., 2002; Sun et al., 2007). As the major class of AMPs, piscidin displays potent broad-spectrum activity against bacteria (Silphaduang & Noga, 2001), fungi (Sung et al., 2008), parasites (Colorni et al., 2008), and even viruses (Chinchar et al., 2004).

Recently, a piscidin-like antimicrobial peptide was isolated from large yellow croaker, and was determined to be a typical gill-expressed peptide distributed in various tissues (Niu et al., 2013). Furthermore, two types of *piscidin-5-like* sequences have been found in large yellow croaker, with their gene structure and sequence characteristics described (Zhou et al., 2014). The large yellow croaker *piscidin-5-like* gene and hybrid striped bass *piscidin-5* gene are reported to be most abundant in the head kidney and intestine, respectively (Salger et al., 2011; Zhou et al., 2014). The synthetic piscidin-4 peptide of hybrid striped bass shows antimicrobial activity against *Staphylococcus aureus*, *Streptococcus iniae*, *Escherichia coli*, and *V. anguillarum* (Noga et al., 2009), and the synthetic piscidin-like peptide of large yellow croaker exhibits broad antimicrobial activity against *S. aureus*, *E. coli*, *Aspergillus niger*, and *Cryptocaryon irritans* in parasitic stages (Niu et al., 2013). These results show the high diversity of piscidin in mRNA expression and function in different fish. However, the effects of teleost piscidin on host defenses against pathogens *in vivo* are still unclear.

In this study, we characterized the cDNA sequence encoding a piscidin-like peptide, *Lcpis5lt3*, from large yellow croaker. Its mRNA expression in different tissues post *V. alginolyticus* infection was studied using quantitative PCR. In addition, the antimicrobial activity of synthetic peptides was also investigated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Fish rearing

Healthy large yellow croaker, without pathological signs and weighing 35–40 g (fish age 7–9 months), were obtained from a commercial farm in Ningbo, China. Each 10 fish were kept in 100 L tanks at 25–27 °C in a recirculating system with filtered sea water. Detection was performed to ensure no bacteria were present in the seawater during the experiment. After acclimating for one week, the fish were used in the experiments described below. All experiments were approved by the Experimental Animal Management Law of China and the Animal Ethics Committee of Ningbo University.

Bacterial challenge

Overnight cultures of *V. alginolyticus* ATCC 17749 were diluted to 1: 100 in Tryptic Soy Broth Medium (TSB) (Sigma, Shanghai, China), grown at 28 °C with shaking, and harvested in the logarithmic phase of growth. The cells were washed, resuspended, and diluted to the appropriate concentration in sterile PBS. Sixteen fish were challenged by intraperitoneal injection with 5×10^6 colony forming units (CFUs) of *V. alginolyticus* (in 100 μL PBS) per fish, and sixteen other fish were injected with 100 μL of PBS per fish as a negative control. The liver, spleen, head kidney, kidney, intestine, muscle, brain, and gill were collected from four fish at each time point at 4, 8, 12, and 24 h post-injection (hpi), as previously reported (Wu et al., 2015), then preserved at -70 °C until examination.

Sequence analysis

Gene sequences used for multiple alignment and phylogenetic analysis are listed in Table 1. The similarity between the obtained sequences with other known sequences was analyzed using BLAST search (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). The cleavage site of signal peptides was predicted by the SignalP4.1 program (http: //www.cbs.dtu.dk/services/SignalP/). Protein analysis was performed using online software on the ExPASy Server (http: //www.expasy.org/tools/). Multiple sequence alignment was analyzed using the ClustalW program (http: //clustalw.ddbj.nig.ac.jp/), and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0 (Tamura et al., 2011).

Quantitative PCR (qPCR)

Changes in mRNA expression of *Lcpis5lt3* following *V. alginolyticus* infection were analyzed by qPCR, as previously described (Lu et al., 2016; Wu et al., 2015). Total RNA was extracted from large yellow croaker tissues using RNAiso reagents (TaKaRa). Gene-specific primers were designed based on the cloned cDNA fragments of Lcpis5lt3, LcIL-1β, LcTNF-α, and LcIL-10 (Table 2). Lcpis5lt3 and two types of piscidin-5-like gene sequences were subjected to nucleotide sequence alignment, and the primers of Lcpis5lt3 were designed in the ORF region of a 153–262 bp portion with low sequence identity. BLAST searching indicated that these primer sequences did not share sequence homology with any known large yellow croaker gene sequence, per the large yellow croaker genome (Wu et al., 2014). As an internal PCR control, primers 18S rRNA F and 18S rRNA R were used to amplify a 200-bp fragment of the housekeeping large yellow croaker 18S rRNA (Lc18S rRNA) gene (Accession No. JN211788.1) (Table

2). QPCR was conducted on an ABI StepOne Real-Time PCR System (Applied Biosystems, USA) using SYBR premix Ex Taq (Perfect Real Time) (TaKaRa) in accordance with the manufacturer's instructions. The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. After amplification, melt curves were obtained by slow heating from 60 °C to 95 °C at 0.1 °C/s, with continuous fluorescence collection, confirming that only our specific product peaks were detected. Fish were kept in eight aquaria. Two fish in each aquarium were biologically repeated twice, and the fish experiment was repeated once, for a total of four biological replicates. The mRNA expression of Lcpis5lt3 was normalized against that of 18S rRNA using the $2^{-\Delta\Delta CT}$ method.

Table 1 Piscidin sequences used for multiple sequence alignment and phylogenetic tree analysis

Species		Gene	GenBank ID
Latin name	English name		
Larimichthys crocea	Large yellow croaker	Piscidin5lt3	KX870851
Larimichthys crocea	Large yellow croaker	Piscidin5lt2	KJ879923
Larimichthys crocea	Large yellow croaker	Piscidin5l	KJ879922
Larimichthys crocea	Large yellow croaker	Piscidin I	EU741827
Oplegnathus fasciatus	Rock bream	Piscidin	AB703274
Epinephelus malabaricus	Malabar grouper	Piscidin1	JX412481
Epinephelus malabaricus	Malabar grouper	Piscidin2	JX412480
Epinephelus coioides	Orange spotted grouper	Piscidin	JQ823163
Epinephelus coioides	Orange spotted grouper	Piscidin I	EU741829
Epinephelus bruneus	Longtooth grouper	Piscidin I	JN216987
Epinephelus bleekeri	Duskytail grouper	Piscidin I	HQ437912
Epinephelus fuscoguttatus	Brown marbled grouper	Piscidin I	GU592793
Epinephelus akaara	Red spotted grouper	Piscidin I	EU741828
Morone chrysops	White bass	Piscidin1	AF394243
Morone saxatilis	Striped bass	Piscidin2	AF394244
M. chrysops × *M. saxatilis*	Hybrid striped bass	Piscidin4	HM596029
M. chrysops × *M. saxatilis*	Hybrid striped bass	Piscidin5	HM596030
Dicentrarchus labrax	European sea bass	Dicentracin	AY303949
Siniperca chuatsi	Mandarin fish	Moronecidin	AY647433

Table 2 Oligonucleotide primers used in this work

Gene	Primer	Sequence (5'-3')	Amplification of length (bp)	Tm (°C)
Lcpis5lt3	Lcpis5lt3 F	ATTGTATCGATCTCGCTGGC	101	58
	Lcpis5lt3 R	CATAATTGGGTGGAAAACGG		55
Lc18S rRNA	Lc18S rRNA F	CTCTTAGCTGAGTGTCCCGC	200	60
	Lc18S rRNA R	ACCTCTAGCGGCACAATACG		60
LcIL-1β	LcIL-1β F	ATCTGGCAAGGATCAGCTCA	108	59
	LcIL-1β R	ACCAGTTGTTGTAGGGGACG		60
LcTNF-α	LcTNF-α F	TGGAGTGGAAGAACGGTCAA	173	59
	LcTNF-α R	GAGAGGTGTGAGGCGTTTCC		61
LcIL-10	LcIL-10 F	CAAGAGCATGAAGCCTCACA	169	58
	LcIL-10 R	GCCCACGGCCTTAAATAGAC		59

Antimicrobial activity assays

The mature peptide of Lcpis5lt3 was chemically synthesized with over 95% purity (GL Biochem, Shanghai, China). The antimicrobial activity was determined against a panel of microorganisms. A micro-dilution assay was used to determine the minimal inhibitory concentration (MIC) of the various agents, as previously described with some modification (Li et al., 2014b). Inhibition was defined as growth lesser or equal to one-half of the growth observed in control wells where no peptide

was added (Douglas et al., 2003). Briefly, serial dilutions of the peptides were made at 100, 50, 25, 12.5, 6.25, 3.125, and 1.563 μg/mL in 96-well microtiter plates. Each well contained 100 μL of a bacterial cell suspension at 1×10^5 CFU/mL and 11 μL of test peptide. After incubating for 24 h at the appropriate temperature, microbial growth was examined. All tests were performed in triplicate and each individual experiment was replicated in quadruplicate. For each series of experiments, PBS was employed as a negative control.

Fish survival assay

Fish were divided into three groups (each containing 16 fish) for survival assay. Fish were injected intraperitoneally (ip) with 5×10^6 CFU/g *V. alginolyticus*. After 30 min, fish received ip injections of 1.0 μg/g Lcpis5lt3 or 0.1 μg/g Lcpis5lt3 of fish weight, while the control group received PBS 30 min post injection. Fish were observed every 24 h for death or moribund state for 8 d.

Bacterial burden in tissues

Three groups, each containing six fish, were ip-injected with *V. alginolyticus* (5×10^6 CFU/g). At 30 min post-infection, the fish received ip injections of different doses of Lcpis5lt3 or PBS, respectively. Fish were sacrificed 12 h after ip injection, and the liver, kidney, spleen, and blood were collected. The tissues from each large yellow croaker were weighed and homogenized in 1 mL of sterile PBS (pH 7.2). Homogenates and blood were serially diluted in sterile PBS (pH 7.2) and then plated onto separate Thiosulfate Citrate Bile Salts (TCBS) agar plates for 18 h at 28 °C. CFUs were then calculated in all plates and multiplied by the dilution factor. Tissue samples were normalized to tissue weight (0.1 g), and blood samples were normalized to blood volume (0.1 mL).

Statistical analysis

All data were described as mean±SEM. Statistical analysis of results was conducted by one-way analysis of variance (ANOVA) with SPSS version 13.0 (SPSS Inc, Chicago, USA). *P*-values of less than 0.05 were considered statistically significant.

RESULTS

***Lcpis5lt3* gene analysis**

Using the liver transcriptome analysis of large yellow croaker, the cDNA sequence of the *Lcpis5lt3* gene was identified by BLAST search and submitted to the DDBJ/EMBL/GenBank databases under accession number KX870851. Computer analysis showed that the large yellow croaker *pis5lt3*, *piscidin-5-like*, and *piscidin-5-like type 2* cDNA sequences contained open reading frames (ORFs) of 264 bp, 213 bp, and 210 bp that encoded an 88, 71, and 70 amino acid peptide, respectively. The peptide of Lcpis5lt3 had an estimated molecular weight (MW) of 9.78 kDa and theoretical isoelectric point (pI) of 8.93. All piscidin-5s were comprised of an N-terminal signal peptide (22 amino acids), a mature peptide (22 amino acids), and a C-terminal prodomain (Figure 1). The deduced cleavage site for the signal peptide was

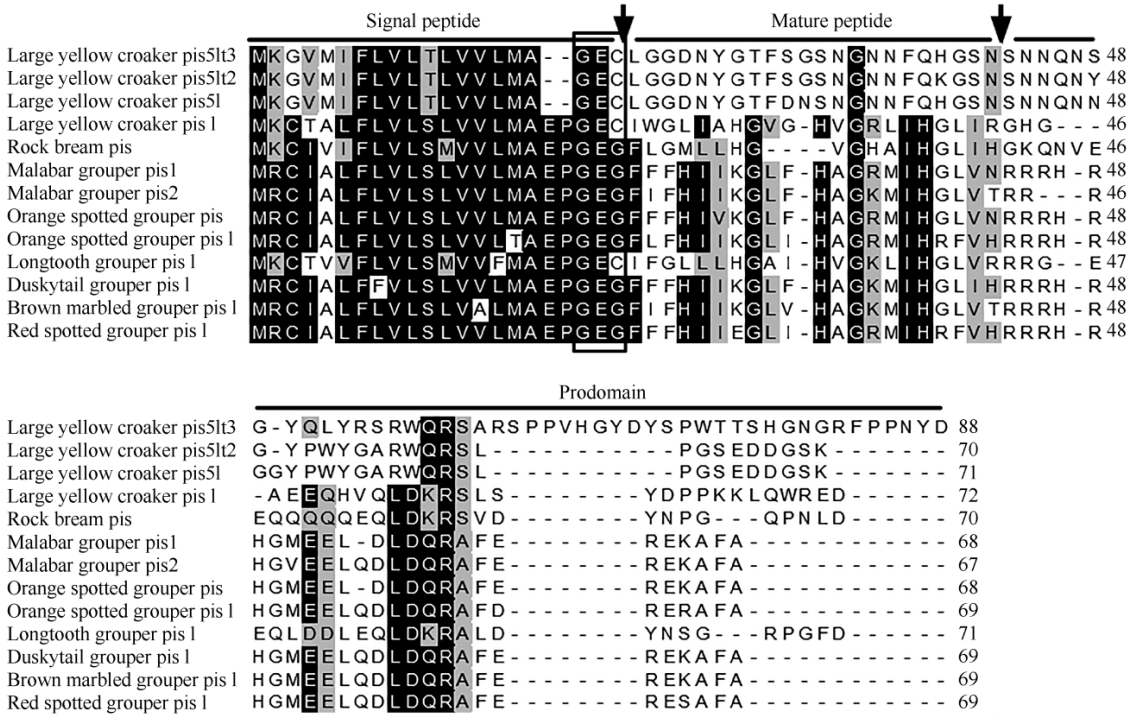

Figure 1 Multiple alignment of the predicted Lcpis5lt3 amino acid sequence with other known piscidins

Similar residues are shaded gray and identical residues are shaded black. The panels show the gaps introduced in the sequences as a dash (-). Predicted cleavage sites for the signal peptide or mature peptide are marked by an arrow (↓). Terminating motifs (GEC, GES, or GEG) for signal peptides are boxed. Accession numbers of sequences are provided in Table 1.

between positions 22 and 23 (GEC-LG), like that of most piscidin sequences referenced, terminating at the motif GEC, GES, or GEG (Figure 1) (Douglas et al., 2003). However, the peptide length of Lcpis5lt3 was longer than that of piscidin-5-like type 2 and piscidin-5-like.

Amino acid sequence alignment of Lcpis5lt3 with closely related sequences revealed conservation in the signal peptide region (Figure 1), but low similarity in the mature peptide and prodomain. In general, Lcpis5lt3 showed low identity (less than 62.5%) to other known piscidin sequences. Based on the known fish piscidin amino acid sequences, a phylogenetic tree was constructed using the neighbor-joining method (Figure 2). Results showed that Lcpis5lt3 and other large yellow croaker piscidin-5s grouped together to form a large yellow croaker piscidin-5 cluster.

Antimicrobial spectrum

The antibacterial activity of the synthesized mature peptide was determined against a panel of microorganisms using the MIC method. The MIC values obtained are reported in Table 3. The synthesized mature peptide of Lcpis5lt3 exhibited activity against *Aeromonas hydrophila*, *V. anguillarum*, *and V. alginolyticus* at 100 μg/mL. It also displayed antibacterial activity with MICs at 50 μg/mL and 6.25 μg/mL against *V. parahaemolyticus* and *S. aureus*, respectively. Lcpis5lt3 had antibacterial activity against *Listeria monocytogenes* with MIC at 3.125 μg/mL. However, this peptide had no effect on *Edwardsiella tarda*, *V. vulnificus*, *V. harveyi*, or *S. iniae* at the concentration tested.

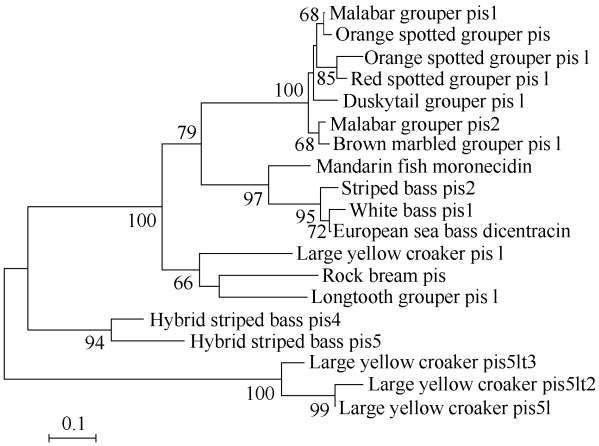

Figure 2 Phylogenetic (neighbor-joining) analysis of the complete amino acid sequences of a piscidin protein using the MEGA5.0 program

The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1 000 replicates; shown only when >60%). The scale bar shows the number of substitutions per base. The sequences used in analysis are listed in Table 1.

Table 3 Antimicrobial activity of synthetic Lcpis5lt3

Bacteria	Strains	Culture medium	Culture temperature (°C)	Lcpis5lt3 MIC (μg/mL)
Edwardsiella tarda	Et-CD	LB	37	-
Aeromonas hydrophila	ATCC7966	LB	37	100
Staphylococcus aureus	ATCC6538	LB	37	6.25
Listeria monocytogenes	ATCC19115	BHI	37	3.125
Vibrio anguillarum	ATCC19264	TSB	28	100
Vibrio alginolyticus	ATCC17749	TSB	28	100
Vibrio vulnificus	ATCC27562	TSB	28	-
Vibrio parahaemolyticus	ATCC33847	TSB	28	50
Vibrio harveyi	ATCC33866	TSB	28	-
Streptococcus iniae	ATCC29178	BHI	37	-

"-" Means no inhibition found at 100 μg/mL.

Constitutive and induced expression in different tissues

QPCR was performed to analyze the temporal expression profile of *Lcpis5lt3* in different tissues of healthy large yellow croaker. The results showed that *Lcpis5lt3* exhibited constitutive expression in all examined tissue, including brain, muscle, liver, intestine, gill, kidney, head kidney, and spleen. The highest expression level of *Lcpis5lt3* was detected in the spleen, followed by the head kidney and kidney (Figure 3A). After *V. alginolyticus* infection, the mRNA expression of *Lcpis5lt3* was rapidly upregulated in liver, head kidney, gill, kidney, and intestine at 4, 8, 12, and 24 hpi, whereas no significant changes were found in the spleen at 4, 8, 12, and 24 hpi (Figure 3B).

Effect of Lcpis5lt3 on the survival rate of *V. alginolyticus*-infected fish

We investigated the bactericidal effects of synthesized mature peptide *in vivo* by monitoring the survival of large yellow croaker infected with *V. alginolyticus* prior to treatment with different concentrations of Lcpis5lt3. All PBS-treated large yellow croaker infected with *V. alginolyticus* died within 7 d after infection. Large yellow croaker treatment with Lcpis5lt3 decreased the mortality rate (Figure 4). At 8 d after *V. alginolyticus* infection, the survival rates were 6% and 50% for large yellow croaker treated with 0.1 μg/g and 1.0 μg/g Lcpis5lt3, respectively (Figure 4).

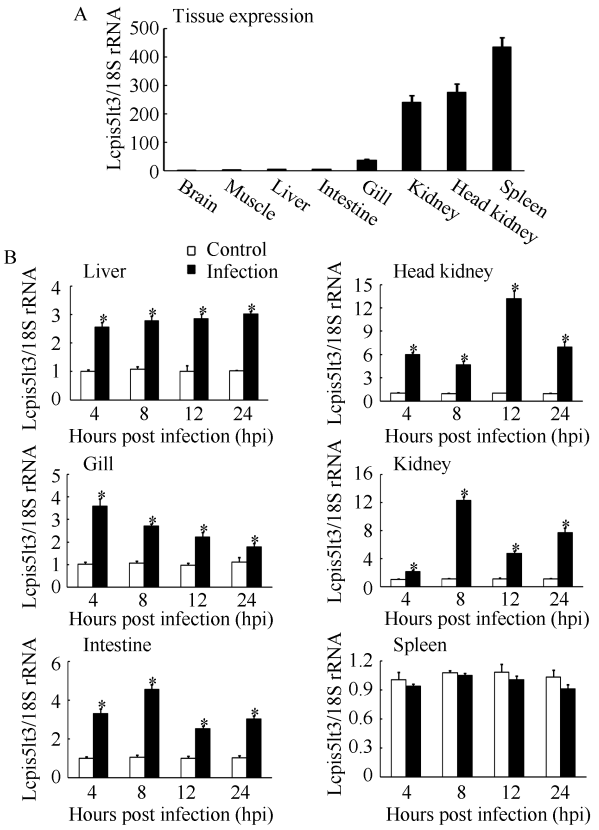

Figure 3 QPCR analysis of Lcpis5lt3 mRNA expression in different tissues

A: Tissues were collected in healthy large yellow croaker. B: Tissues were collected at different time points after *V. alginolyticus* challenge. Fish were injected intraperitoneally with *V. alginolyticus* at 4, 8, 12, and 24 hpi. Lcpis5lt3 transcript levels were normalized to Lc18S rRNA. Data are expressed as mean±SEM. *n*=4. *: *P*<0.05.

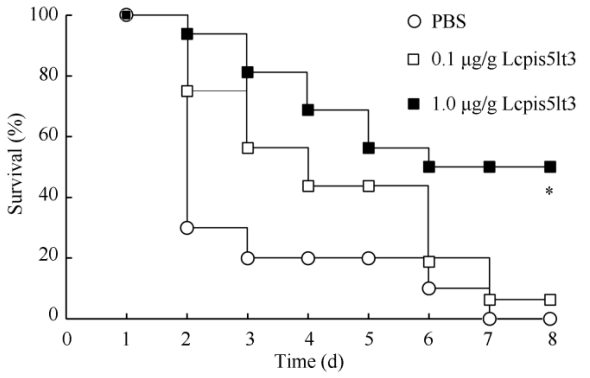

Figure 4 Effect of Lcpis5lt3 on the survival rate of large yellow croaker

Fish were ip-injected with *V. alginolyticus* (5×10^6 CFU/g). After 30 min, 0.1 μg/g or 1.0 μg/g Lcpis5lt3 was ip-injected into fish, respectively. The control group received an equal volume of PBS. Fish were monitored for signs of sickness and mortality every 24 h for 8 d. Experiments are representative of 16 animals per group. *: *P*<0.05.

Bacterial burden in tissues and blood

To examine the impact of the synthesized mature peptide of Lcpis5lt3 on bacterial proliferation and dissemination *in vivo*, the bacterial loads were quantitated in the liver, spleen, kidney, and blood following ip-injection with 0.1 μg/g Lcpis5lt3 or 1.0 μg/g Lcpis5lt3 in *V. alginolyticus*-challenged fish. Fish treated with 0.1 μg/g Lcpis5lt3 and 1.0 μg/g Lcpis5lt3 all showed a reduction in *V. alginolyticus* load in the liver, spleen, kidney, and blood 12 hpi after *V. alginolyticus* challenge in comparison with the PBS-treated control group (Figure 5). There were significant differences between the control and 1.0 μg/g Lcpis5lt3 groups in all tested tissues, whereas the 0.1 μg/g Lcpis5lt3-treated group showed only small variation (Figure 5).

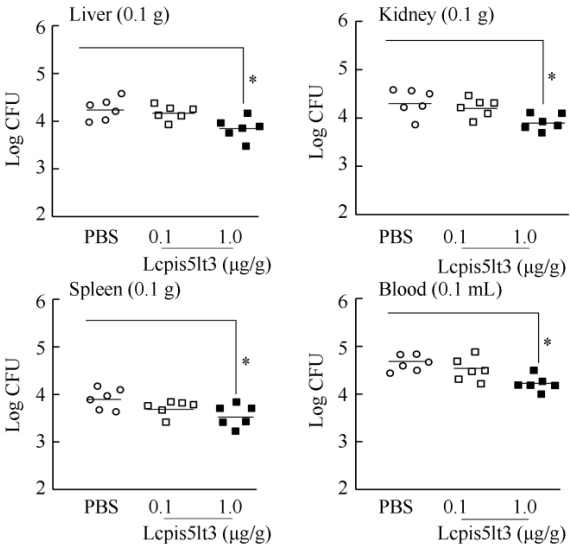

Figure 5 Effect of Lcpis5lt3 on bacterial burden in large yellow croaker liver, spleen, kidney, and blood

Fish were ip-injected with live *V. alginolyticus* at a dose of 5×10^6 CFU/g and received the same volume of 0.1 μg/g or 1.0 μg/g Lcpis5lt3 30 min after injection. The control group received an equal volume of sterile PBS. Fish were euthanized 12 h later, and the liver, spleen, kidney, and blood were collected. *n*=6 in each group. *: *P*<0.05 versus the PBS-treated group.

Effect of Lcpis5lt3 on cytokine expression following infection

To explore the effect of Lcpis5lt3 on inflammatory gene expression *in vivo*, the mRNA levels for inflammatory cytokines LcTNF-α, LcIL-1β, and LcIL-10 were evaluated in tissues collected from the fish after Lcpis5lt3 treatment and PBS-treated controls following infection with *V. alginolyticus*. QPCR analysis revealed a significant decrease in the expressions of LcTNF-α, LcIL-1β, and LcIL-10 transcripts in the tissues of fish treated with 0.1 μg/g or 1.0 μg/g Lcpis5lt3 compared with fish treated with PBS (Figure 6).

DISCUSSION

In the present study, *Lcpis5lt3* was identified and characterized

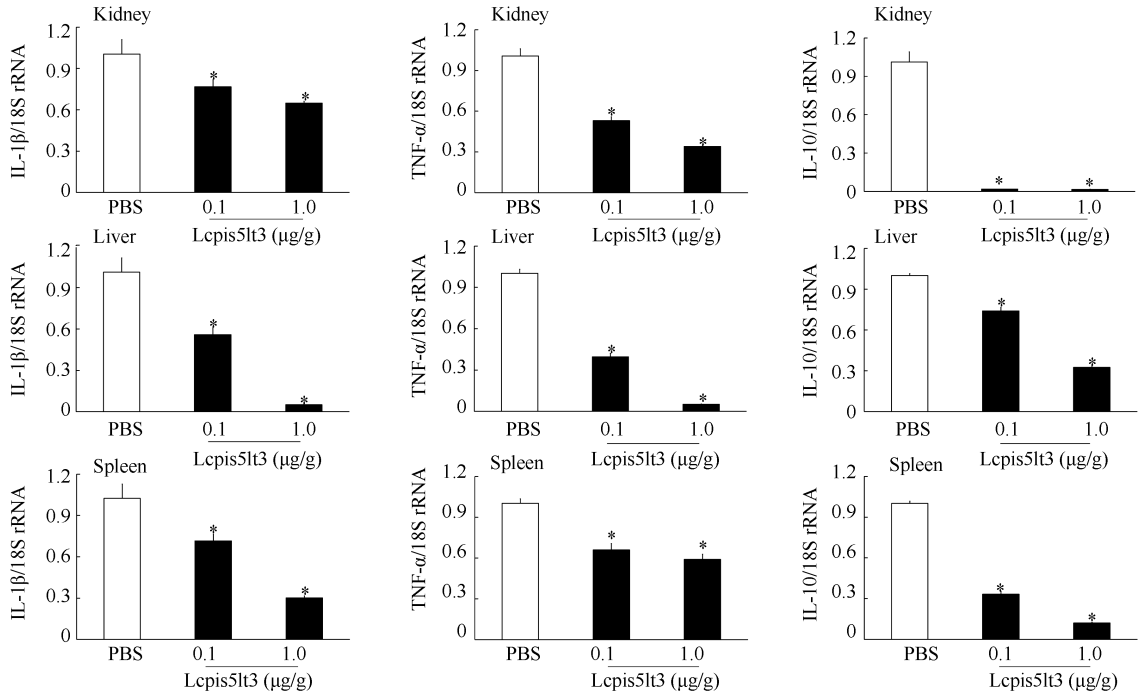

Figure 6 Effect of Lcpis5lt3 on mRNA levels of LcTNF-α, LcIL-1β, and LcIL-10

Fish were ip-injected with *V. alginolyticus* at a dose of 5×10^6 CFU/g and separately received the same volume of 0.1 μg/g or 1.0 μg/g Lcpis5lt3 30 min after infection. LcTNF-α, LcIL-1β, and LcIL-10 mRNA levels were evaluated at 12 hpi and were normalized by Lc18S rRNA content. *: $P<0.05$ versus the PBS-treated group.

in large yellow croaker. Lcpis5lt3 comprised a signal peptide, mature peptide, and prodomain, as shared by other piscidin paralogues (Buonocore et al., 2012; Niu et al., 2013). Similar to other piscidins (Zhou et al., 2014), Lcpis5lt3 showed more conservation in the signal peptide and less conservation in the mature peptide and prodomain. The phylogenetic tree confirmed that Lcpis5lt3 was grouped together with large yellow croaker piscidin-5-like type 2 and piscidin-5-like to form a cluster. This result revealed that large yellow croaker pis5lt3 was a variation type of the piscidin-5-like peptide. According to previous research, several piscidin paralogues can be found in a single fish species. For example, piscidin-4 and piscidin-5 genes are found in hybrid striped bass (Salger et al., 2011); piscidin-1, piscidin-2, piscidin-3, piscidin-4, and piscidin-5 genes are found in tilapia (Peng et al., 2012); and piscidin-1, piscidin-2, and piscidin-2-β genes are found in Atlantic cod (Ruangsri et al., 2012). There are also several piscidin genes found in large yellow croaker, such as piscidin-like, piscidin-5-like, and piscidin-5-like type 2 (Zhou et al., 2014). These results show a high diversity of piscidin in different fish.

The transcripts of piscidin genes are widely distributed in various tissues (Buonocore et al., 2012; Salger et al., 2011). In rock bream, the piscidin gene is highly expressed in the gills of healthy fish (Bae et al., 2016). In tilapia, piscidin-2 is abundant in the skin, head kidney, and spleen; piscidin-3 is abundant in the skin, head kidney, and gill; and piscidin-4 is abundant in the intestine (Peng et al., 2012). In the large yellow croaker, the piscidin-like gene is most abundantly expressed in the gill of unchallenged fish (Niu et al., 2013), and the piscidin-5-like is most abundant in the head kidney (Zhou et al., 2014). In this study, the *Lcpis5lt3* transcript was highly expressed in the spleen, head kidney, and kidney of the large yellow croaker, suggesting that piscidin genes have a variety of tissue expression patterns. In mandarin fish, piscidin gene mRNA expression is upregulated in the intestine, spleen, kidney, liver, skin, and gill after stimulation with LPS (Sun et al., 2007). In large yellow croaker, piscidin-like gene mRNA expression is significantly upregulated in the gill, skin, spleen, head kidney, liver, and intestine after *C. irritans* infection (Niu et al., 2013). In the current study, the mRNA expression levels of *Lcpis5lt3* were upregulated in the liver, head kidney, gill, kidney, and intestine after *V. alginolyticus* infection. These results suggest that most fish piscidin in a variety of tissues were upregulated after infection. Furthermore, we found there were no significant changes in *Lcpis5lt3* in the spleen after infection. However, this mechanism needs further investigation.

Unique for the field of fish research, AMPs have potential applications to prevent pathogenic microbes in aquaculture (Masso-Silva & Diamond, 2014). The constant risk of large-scale microbial infection that can lead to significant economic losses requires new strategies to prevent or treat these pathogens (Masso-Silva & Diamond, 2014). In rock bream, the synthetic piscidin peptide exhibits antimicrobial activity against *E. tarda*, *V. vulnificus*, *V. harveyi*, and *S. iniae* (Bae et al., 2016). In tilapia, five synthetic piscidin peptides have been shown to exhibit antimicrobial activity against *V. vulnificus*, *V. alginolyticus*,

A. hydrophila, and *Pseudomonas aeruginosa* (Peng et al., 2012). In contrast, our results showed that Lcpis5lt3 had no effect on *E. tarda*, *V. vulnificus*, *V. harveyi*, or *S. iniae* at the antimicrobial activity tested. However, Lcpis5lt3 exhibited antimicrobial activity against *L. monocytogenes*, *S. aureus*, *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, and *A. hydrophila in vitro*. These results demonstrate that Lcpis5lt3 possesses broad-spectrum antimicrobial activity, and different piscidins in teleosts possess spectrum variety in antimicrobial activity.

To evaluate the antimicrobial activity of Lcpis5lt3 against *V. alginolyticus in vivo*, we performed the survival rate assay. Our results showed that the synthesized mature peptide of Lcpis5lt3 can increase the survival rate of fish infected with *V. alginolyticus* at different concentrations. The overall survival rate of the 1.0 μg/g Lcpis5lt3 group was higher than that of the 0.1 μg/g Lcpis5lt3 group after infection; this effect was accompanied by a lower bacterial burden and a decline in LcTNF-α, LcIL-1β, and LcIL-10 mRNA expression. The cytokines TNF-α and IL-1β in teleosts are powerful proinflammatory cytokines released by several immune cells during infection or tissue damage and are involved in a diverse range of inflammatory and infectious conditions (Roca et al., 2008; Seppola et al., 2008; Wu et al., 2015). However, IL-10 is a critical anti-inflammatory cytokine, whose expression is induced after proinflammatory mediators. It helps control immune responses and thereby minimize tissue damage (Secombes et al., 2011; Wang and Secombes, 2013; Zhu et al., 2013). As an autoregulatory mediator, IL-10 has important regulatory effects on immunological and inflammatory responses due to its capacity to inhibit the production of proinflammatory cytokines by monocytes (Ringø, 2011). In this study, we found that LcTNF-α, LcIL-1β, and LcIL-10 mRNA expression were all downregulated in the 0.1μg/g and 1.0 μg/g Lcpis5lt3 groups after infection, suggesting that inflammation in fish after *V. alginolyticus* infection was relieved. Similar results were found with tilapia piscidin-4 (TP4). For example, mice treated with TP4 resulted in the downregulation of TNF-α, IL-1β, and IL-10 (Narayana et al., 2015). However, it is still unclear whether piscidins can directly downregulate inflammatory cytokines, such as the AMPS of other fish (Chen et al., 2016; Li et al., 2015), or only kill pathogens to reduce the expression of inflammatory cytokines. Further investigation is needed to elucidate the role of Lcpis5lt3 in the immune responses of large yellow croaker after infection.

In conclusion, we characterized *Lcpis5lt3* as a member of the piscidin family in large yellow croaker. Results showed that *V. alginolyticus* infection led to the alteration of *Lcpis5lt3* mRNA expression in different tissues. Antimicrobial assays *in vitro* and *in vivo* also showed that the synthetic mature peptide had broad spectrum antimicrobial activity *in vitro* and increased fish survival upon bacterial infection *in vivo*. These data provide new insights into the innate immunity of large yellow croaker against pathogens and reveal the value of piscidin as a therapeutic agent to control microbial infections.

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ZOOLOGICAL RESEARCH

Re-evaluating data quality of dog mitochondrial, Y chromosomal, and autosomal SNPs genotyped by SNP array

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ABSTRACT

Quality deficiencies in single nucleotide polymorphism (SNP) analyses have important implications. We used missingness rates to investigate the quality of a recently published dataset containing 424 mitochondrial, 211 Y chromosomal, and 160 432 autosomal SNPs generated by a semicustom Illumina SNP array from 5 392 dogs and 14 grey wolves. Overall, the individual missingness rate for mitochondrial SNPs was ~43.8%, with 980 (18.1%) individuals completely missing mitochondrial SNP genotyping (missingness rate=1). In males, the genotype missingness rate was ~28.8% for Y chromosomal SNPs, with 374 males recording rates above 0.96. These 374 males also exhibited completely failed mitochondrial SNPs genotyping, indicative of a batch effect. Individual missingness rates for autosomal markers were greater than zero, but less than 0.5. Neither mitochondrial nor Y chromosomal SNPs achieved complete genotyping (locus missingness rate=0), whereas 5.9% of autosomal SNPs had a locus missingness rate=1. The high missingness rates and possible batch effect show that caution and rigorous measures are vital when genotyping and analyzing SNP array data for domestic animals. Further improvements of these arrays will be helpful to future studies.

Keywords: SNP array; Dog; Mitochondrial; Y chromosomal; Autosomal

INTRODUCTION

Single-nucleotide polymorphism (SNP) arrays have received wide recognition for detecting DNA polymorphisms in domestic animals (Goddard & Hayes, 2009). The availability of SNP arrays to incorporate not only dense autosomal markers, but also hundreds of mitochondrial and Y chromosomal SNPs, greatly assists breeding and population history inferences (Shannon et al., 2015b). Genotyping SNPs offers superior efficiency and convenience compared with traditional Sanger sequencing or genotyping techniques, such as denaturing high-performance liquid chromatography (DHPLC) and SNPshot. Like other high-throughput techniques, however, SNP assays are not infallible. Difficulties can arise from diverse, complex, and often cryptic sources, and different factors can converge to produce an artifact (Pompanon et al., 2005). With new technological advancements in the genotyping landscape, some potential artifacts remain unknown, untested, or unaccounted for (Leek, 2014; Leek et al., 2010). Previous studies on human populations have established potential technological and experimental pitfalls in genotyping, which could compromise data quality (Palanichamy & Zhang, 2010; Peng et al., 2014). To investigate these issues in domestic animals, we performed an independent re-evaluation of recently published SNP array data representing a global dog population (Shannon et al., 2015b).

MATERIALS AND METHODS

We retrieved dog SNP datasets from Dryad (doi: 10.5061/dryad.v9t5h) (Shannon et al., 2015a). Detailed methodology is described elsewhere (Shannon et al., 2015b). Briefly, DNA was extracted predominantly from whole blood samples by salt precipitation from 4675 pure breed, 168 mixed breed, and 549 village dogs, plus 14 grey wolves (Supplementary Table S1). The samples were genotyped against 424 mitochondrial, 211 Y chromosomal, and 160 432 autosomal SNP markers using a

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semicustom Illumina SNP array (Shannon et al., 2015b). We used PLINK v.1.07 to determine the missingness rates (MRs) of the datasets (Purcell et al., 2007). We analysed all individual MRs (iMR) for both mitochondrial and autosomal marker types, except for the Y chromosomal marker in females. We also calculated the locus MR (lMR) to assess the MRs for all SNPs. We used IBM SPSS statistics version 20.0 (SPSS, Inc., Chicago, IL, USA) for data analysis, and box plots were drawn by BoxPlotR software (Spitzer et al., 2014).

RESULTS

Full iMR and lMR results are shown in Supplementary Tables S2 and S3, respectively. As summarized in Figure 1, complete genotyping (MR=0) for mitochondrial and Y chromosomal SNPs was observed for 3 039 (56.2%) and 1 896 (71.2%) individuals, respectively, with 980 (18.1%) and 107 (4.0%) individuals completely missing genotyping (MR=1) for the two marker types, respectively. Pure breed dogs tended to have a higher iMR (1) than that of other dogs. Additionally, overall mean iMR values were generally higher in pure breed dogs and much higher in grey wolves, specifically for mitochondrial and Y chromosomal marker types (Table 1). This trend was mirrored in the mean iMR across breeds, excluding MR=0 values (Supplementary Table S4). All individuals recorded autosomal genotyping iMR >0 to <0.5. Combined analysis of all MR values >0 and <1 (Figure 2) showed a higher mean iMR for the Y chromosomal (>40%) than the other two markers.

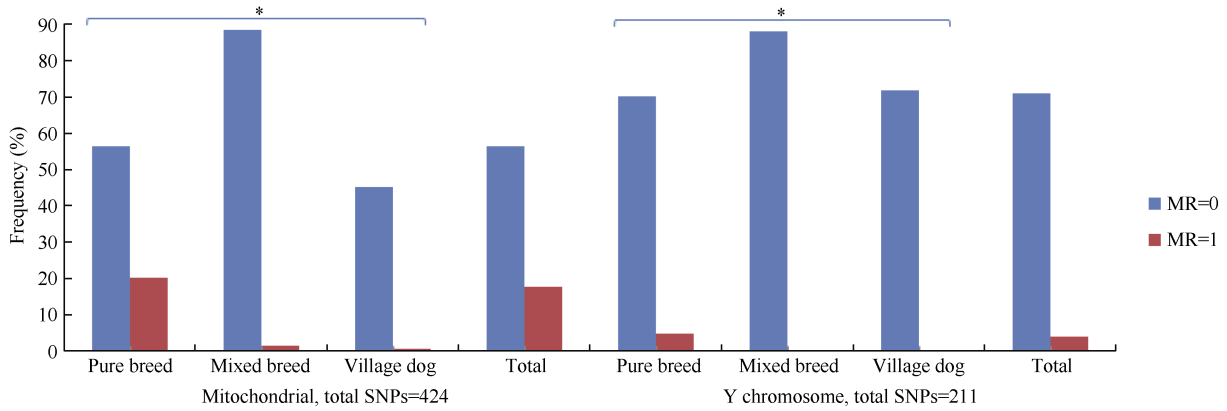

Figure 1 Individual missingness rates (iMR) for mitochondrial and Y chromosomal marker types

Complete genotyping (MR=0), completely missed genotyping (MR=1). Autosomal markers had neither MR categories and were not plotted. *: $P<0.05$.

Table 1 Comparison of individual missingness rates (iMR) across different breed categories

	Overall iMR, mean (standard deviation)		
	Mitochondrial	Y chromosomal	Autosomal
Pure breeds	0.206 1 (0.403 4)	0.164 6 (0.365 5)	0.028 2 (0.059 9)
Mixed breeds	0.018 1 (0.132 8)	0.012 5 (0.104 9)	0.003 2 (0.014 9)
Village dogs	0.011 2 (0.085 0)	0.008 (0.053 3)	0.004 1 (0.013 6)
Grey wolves	1	0.966 8	0.130 3 (0.017 0)
ANOVA P value	0.000 1	0.000 1	0.000 1

Overall genotype missingness rates (MR>0) for mitochondrial and Y chromosomal SNPs were realized in 2 367 (43.8%) and 766 (28.8%) individuals, respectively, with the missing genotyping proportions in each breed summarized in Supplementary Table S4. Of the 980 individuals with mitochondrial MR=1, 374 were males, which all had Y chromosomal MR>0.96 (Figure 1 and Supplementary Table S5). The mean autosomal MR was also significantly higher for these 374 males (0.135) compared with the other 2 288 males (0.002) (Table 2). Further scrutiny indicated that all 980 individuals with mitochondrial MR=1 came from 1 325 samples that had a different experimental format, given the assaying plate numbering system (Sample IDs prefix, Supplementary Table S2). There was a marked difference in mean iMR across all three marker types between the two classes of samples, with those undergoing assaying plate serialization bearing lower missed genotyping rates (Supplementary Table S6). These observations suggest a likely batch effect (Leek, 2014; Leek et al., 2010) in the case of the 374 males.

Assessment of lMR showed that none of the mitochondrial or Y chromosomal SNPs achieved complete genotyping (MR=0). While 5.9% of the autosomal SNPs were completely genotyped, 0.5% of the autosomal SNPs together with 0.7% of the mitochondrial SNPs had a ⩾20% MR among the study individuals (Table 3). Overall, lMR was higher for mitochondrial and Y chromosomal SNPs compared with that for autosomal SNPs (Figure 3).

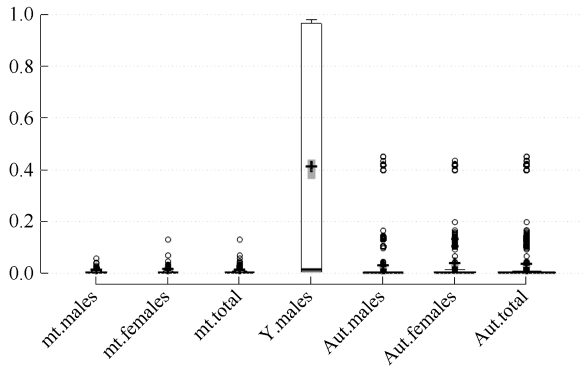

Figure 2 Box plot showing the individual missingness rates (iMR) for mt: mitochondrial (*n*=1 387), Y: Y chromosomal (*n*=659), and Aut: autosomal (*n*=2 744) marker types according to gender for the 0<MR<1 category

Vertical axis represents iMR scores, center lines show the medians, box limits indicate the 25th and 75th percentiles, Tukey whiskers extend to minimum and maximum MR values, and crosses represent sample means.

DISCUSSION

The missingness rate can be used to clarify overall quality of genotyping. Problems at any stage of the genotyping process can adversely impact data analyses, including the definition of haplotypes and calculation of genetic diversities. Missingness rates can inform decisions on how to account for possible errors to support the genotyping process, and possibly inform technological advancements in SNP arrays (Laframboise, 2009). The observed overlapping pattern of high MR statistics for mitochondrial and Y chromosomal SNPs among the 374 males represents a possible batch effect scenario (Leek et al., 2010).

Batch effects commonly occur in high-throughput technologies, where a subgroup of observations show qualitatively different behaviors across conditions, which might not be related to biological variability (Leek et al., 2010). Batch effects, like other genotyping problems, arise from ubiquitous sources that are often not fully recorded or reported, ranging from sample/DNA competence, date/time of experiment, technician input, reagents, chip numbers, as well as platforms or instruments used (Leek, 2014; Leek et al., 2010; Pompanon et al., 2005). Full experimental records and individual sample information, as highly advocated elsewhere (Kitchen et al., 2010; Leek et al., 2010), play vital roles in facilitating re-evaluations or meta-analyses of multiple datasets. This was a limitation encountered in our analysis, which lowered the power for definitive validation of the suspected batch effect and factors underlying high MR values.

In the present study, MRs tended to be higher for pure breed dogs than for other dogs, suggesting potential breed-based differential SNP array missingness, contrary to more robust technologies such as next-generation sequencing. Missing genotype calls are widespread in high-throughput genotyping, but their effect on subsequent analyses has been largely ignored (Fu et al., 2009; Yu, 2012). In SNP arrays, missing call rates arise from technical issues like SNP array manufacturing, DNA processing, batch size and composition, or genotype calling criteria, as well as biological issues such as previously uncharacterized variants or DNA quality and quantity (Didion et al., 2012; Fu et al., 2009; Hong et al, 2008.; Nishida et al., 2008). In addition to careful DNA quality control and quantity standardization, other mitigation measures to reduce high MRs should include employing large and uniform batch sizes in genotype calling, using homogenous samples in the same batches (Hong et al., 2008), reviewing the suitability of quality control filtering cutoffs when calling genotypes (Fu et al., 2009), and continuous characterization and inclusion of rarer genomic variants in array designs (Didion et al., 2012).

Table 2 Comparison of individual missingness rates (iMR) for 374 males with likely batch effect versus remaining males

	Mean MR (Standard deviation)					
	Mitochondrial SNPs	*P*-value	Y chromosomal SNPs	*P*-value	Autosomal SNPs	*P*-value
In*	1 (0)	0.001	0.982 (0.013)	0.001	0.135 (0.069)	0.001
Out*	0.001 (0.003)		0.002 (0.007)		0.002 (0.003)	

*In=374 male individuals with likely batch effect (mitochondrial SNPs MR=1 and Y chromosomal SNPs MR>0.96), Out=other individuals (*n*=2 288).

Table 3 Summary of locus missingness rates (lMR) for mitochondrial, Y chromosomal, and autosomal SNPs

	Genotyping marker, No. SNPs (%)			Total
	Mitochondrial SNPs	Autosomal SNPs	Y chromosomal SNPs	
SNP MR				
MR=0	0	9 486 (5.9)	0	9 486 (5.9)
0<MR<0.1	0	134 747 (84.0)	4 (1.9)	134 751 (83.7)
0.1=<MR<0.2	421 (99.3)	15 456 (9.6)	207 (98.1)	16 084 (10.0)
0.2=<MR<0.3	3 (0.7)	743 (0.5)	0	746 (0.5)
Total	424 (100.0)	160 432 (100.0)	211 (100.0)	161 067 (100.0)

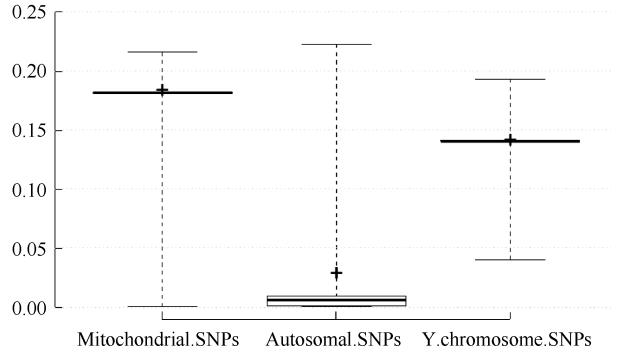

Figure 3 Box plot showing the locus missingness rates (IMR) for mitochondrial, Y chromosomal, and autosomal SNPs MR>0

Vertical axis represents iMR scores, center lines show the medians, box limits indicate the 25th and 75th percentiles, spear whiskers extend to minimum and maximum MR values, and crosses represent sample means.

Due to the diverse, complex, and cryptic nature of genotyping issues in high-throughput technologies, such as batch effects, a thorough understanding and awareness of potential causal avenues, consequences, and mitigation strategies are serious concerns among researchers (Kitchen et al., 2010; Kupfer et al., 2012; Leek, 2014; Leek et al., 2010; Palanichamy & Zhang, 2010). SNP array technology, computational methodology, and biological inferences are closely interlinked (Laframboise, 2009). Our findings, therefore, point to the necessity of rigor and caution in the generation and use of SNP array genotyping data for domesticated animals, especially those improved for specialized traits. Continuous robustification and extensive pre-commercialization qualification of SNP arrays are areas for future consideration.

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AVAILABILITY OF DATA AND MATERIALS

All data and software used in this paper are freely available. The SNP dataset for the 5406 dog samples has been published previously (Shannon et al., 2015b), and is freely available at: http: //www.datadryad.org/resource/doi: 10.5061/dryad.v9t5h. Both the PLINK and BoxPlotR software are freely available at: http: //pngu.mgh.harvard.edu/~purcell/plink and http: //boxplot.tyerslab.co/, respectively. In addition, we have provided the complete missingness rate data in the online version of this article in Supplementary Table S2 (results of individual missingness rates) and Supplementary Table S3 (results of locus missingness rates) plus other supplementary results supporting this paper.

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Meeting report: the 4th symposium on animal models of non-human primates in Kunming, Yunnan, China

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From 2 to 4 November, 2016, the 4th Symposium on Animal Models of Non-Human Primates (NHP) was held in Kunming, Yunnan, China. This meeting was organized by the Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences (CAS) & Yunnan Province, Kunming Primate Research Center (KPRC), *Zoological Research*, and Kunming Institute of Zoology (KIZ), CAS.

The agenda was led by a keynote lecture from Dr. Yong-Gang Yao, the director of KIZ and chair of the symposium. Presentations from 18 invited speakers and eight local speakers from KIZ were delivered under four topic sessions: “Infectious Diseases and Vaccines”, “Neuroscience and Neurological Disorders”, “Stem Cell and Transgenic NHP Models” and “NHP Animal Welfare and Others”. Another highlight of the symposium was the tour of the KPRC organized by Drs. Yong-Tang Zheng and Jia-Li Li. Since its establishment in 2005, the KPRC has become a highly-regarded NHP research and development center, serving as one of the major bases for biomedical research in China.

As emphasized in the symposium, NHP animal models are not only irreplaceable in basic research, but also of critical importance in a wide range of scientific and clinical investigations. The symposium, together with the previous series (Yao et al., 2015), also provided opportunities to promote communication and facilitate collaboration among researchers in the field of NHP studies, from basic research to translational medicine.

KEYNOTE LECTURE

Following a warm welcome by **Dr. Yong-Tang Zheng** (KIZ, CAS), the co-chair of the symposium, **Dr. Yong-Gang Yao** (KIZ, CAS) delivered the keynote lecture on the establishment of the National Research Facility for Phenotypic and Genotypic Analysis of Model Animals (Primates) (NRFPGAMA) and its related research programs on phenotypic and genetic studies. In it, the blueprint of the NRFPGAMA was presented, which highlighted its involvement in sophisticated crosstalk from NHP breeding, NHP resource management, mega data collection and analysis, and relevant applications in various research fields and drug developments. Also discussed were the future directions of this major, multifaceted scientific facility, which will serve as much more than a traditional research platform. Its potential applicable purposes will be greatly extended, and it will continue to be actively involved in coordinating multi-disciplinary research and application in biomedical studies and drug development, as well as to lead the future primate related researches.

FEATURED TOPIC SESSIONS

Infectious diseases and vaccines

Dr. Zhi-Wei Chen (AIDS Institute, University of Hong Kong) described his group’s pioneering studies to elucidate the mucosal seeding of SARS-CoV infection using the Chinese macaque model (Liu et al., 2016).

Dr. Xia Jin (Institute Pasteur of Shanghai, CAS) presented research entitled “Dengue and Zika rhesus monkey model in the development of vaccines”, in which the history and current applications of rhesus macaque (*Macaca mulatta*) models in infectious disease and vaccine evaluations of dengue and Zika viruses were introduced (Dudley et al., 2016; Liang et al., 2015; Logan, 2016). Dr. Jin also presented work on the development of subunit dengue vaccines and the use of NHP for the evaluation of vaccine conferred protection (Cheng et al., 2016; Jin et al., 2015;).

Dr. Xiao-Ping Chen (Guangzhou Institutes of Biomedicine and Health, CAS) reported on a rhesus macaque model of co-infection with malaria and simian immunodeficiency virus (SIV), along with antiretroviral therapy (ART) treatment. Dr. Chen showed that *Plasmodium* infection reduced the replication-competent virus pool in resting $CD4^+$ T cells (a major viral reservoir), which might be attributable to the activation and apoptosis of memory $CD4^+$ T cells induced by malaria, with histone acetylation and NF-κB activation in resting $CD4^+$ T cells also important in this reduction. Therefore, because more HIV-

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1-infected individuals in malaria-endemic areas are receiving ART treatment, clinicians should determine whether some patients co-infected with *Plasmodium* are experiencing virological benefits (Zhan et al., 2014).

Dr. Yong-Tang Zheng (KIZ, CAS) delivered a presentation entitled "Basic biological research of northern pig-tailed macaques (*Macaca leonine*, PTMs) and its application in AIDS models". Dr. Zheng obtained 250 PTMs from Vietnam, and analyzed their hematology and blood chemistry parameters, immunoglobulin, complements and CRP levels, immune cells in the peripheral blood mononuclear cells (PBMCs), classical MHC class I genes, and pathogens. The physiological and biochemical indexes of PTMs suggested that, compared with Chinese rhesus macaques, PTMs are genetically closer to humans and thus might serve as a better NHP experimental animal (Lei et al., 2014; Lian et al., 2016; Pang et al., 2013; Zhang et al., 2014, 2016; Zheng et al., 2014; Zhu et al., 2015). Based on these findings, Dr. Zheng's team inoculated PTMs with HIV-1 strains and monitored the infections for three years. The results suggest that HIV-1-infected NPMs might function as a potential NHP animal model in HIV-1 latency studies and in developing novel therapeutic strategies.

Dr. Bo Zhang (Wuhan Institute of Virology, CAS) discussed their recent study on the isolation of the Chikungunya virus (CHIKV) and the establishment of its reverse genetics platform. CHIKV is a member of the *Alphavirus* genus. It is an important mosquito-borne human pathogen, which can cause abrupt high fever, headache, rashes, myalgia, and arthralgia, with arthritis possibly lasting for months or years. So far, however, no effective antiviral drugs or vaccines against CHIKV infection are available. Dr. Zhang's team recently isolated a CHIKV strain (GenBank Accession No. KC488650) from a clinically CHIKV-positive patient in China through serial passages in C6/36 cells, and found that it belonged to the Asian lineage of CHIKV. Using this isolated strain, an infectious clone of CHIKV and a reporter virus (eGFP-CHIKV) with an eGFP gene were constructed and used to investigate viral replication through measuring eGFP expression level. The eGFP-CHIKV reporter virus was then used to identify inhibitors and screen neutralizing antibodies against CHIKV (Deng et al., 2016).

Dr. Ling Chen (Guangzhou Institutes of Biomedicine and Health, CAS) discussed his group's work on the preparation of neutralized monoclonal antibodies (mAbs) using immunized macaques to against outbreaks of highly infectious diseases (Meng et al., 2013; Pan et al., 2014).

Dr. Jian-Qing Xu (Fudan University) presented work in the sequential immunization strategy of heterologous HIV immunogens (Zhou et al., 2016).

Neuroscience and neurological disorders

Dr. Bao-Ming Li (Nanchang University) provided evidence that myelination of the prefrontal cortex (PFC) is essential for the development of projection neurons in the PFC. His group showed that adverse early-life experience, i.e., deprivation of parental relationships, can induce permanent phenotypic changes and impair the cognitive functions associated with PFC. Myelination is necessary for medial PFC (mPFC)-dependent behaviors. Blockade of oligodendrocyte (OL) differentiation or lysolecithin-induced demyelination can impair mPFC functions. Histone deacetylases 1/2 (HDAC1/2) were drastically reduced in neonatal maternal separation (NMS) rats; the inhibition of HDAC1/2 promoted Wnt activation, which negatively regulated OL development, whereas, the selective inhibition of Wnt signaling by XAV939 partly rescued myelination arrestment and behavior deficiency caused by NMS. These findings indicate that, to some extent, NMS impairs mPFC cognitive functions through modulation of oligodendrogenesis and myelination (Yang et al., 2016).

Dr. Xiao-Zhong Peng (Chinese Academy of Medical Sciences) presented work on the roles of non-coding RNAs in neurodevelopmental disorders, and showed that microRNAs (miRNAs) in the development of brain and non-coding molecules might assist in the development and function of the central nervous system and drive neurodevelopmental disorders (Lin et al., 2016).

Dr. Xiao-Qing Zhang (Tongji University) described the mechanisms and determinations of neuronal fate during early neural development and their evolutionary interpretation. The anteroposterior patterning of the central nervous system follows an activation/transformation model, suggesting that a prospective telencephalic fate will be activated by default during the neural induction stage, with the anterior fate transformed posteriorly per caudalization morphogens. Although both extrinsic signals and intrinsic transcription factors have been implicated in dorsoventral (DV) specification of vertebrate telencephalon, the DV patterning model remains elusive. Dr. Zhang's team assumed that human forebrain DV patterning also follows an activation/transformation paradigm. Human neuroectoderm (NE) will activate forebrain dorsal fate automatically and this default anterior dorsal fate does not depend on Wnts activation or Pax6 expression. Forced expression of Pax6 in human NE hinders its ventralization, even under sonic hedgehog (Shh) treatment, suggesting that dorsal genes repress the ventral fate. Genetic manipulation of Nkx2.1, a key gene for forebrain ventral progenitors, showed that Nkx2.1 is neither necessary nor sufficient for Shh-driven ventralization. Dr. Zhang's study proposed that Shh represses dorsal genes of human NE and subsequently transforms the primitively activated dorsal fate ventrally in a repression release manner (Chi et al., 2016).

Dr. Liang Wang (Beijing Institute of Psychology, CAS) discussed the electrophysiological basis and function of the human brain connectome. Using standard fMRI paradigms, Dr. Wang's group identified 25 topographic maps in a large population of individual subjects and transformed them into either surface- or volume-based standardized spaces. By evaluating the topographic organization across the whole visual cortex, novel information about the organization of individual visual field maps and large-scale biases in visual field coverage were provided, with each atlas for use with independent subjects then validated. The probabilistic atlases quantified the variability of topographic representations in the human cortex, and thus provide a useful reference for comparing data across studies that can be transformed into these standard spaces

(Wang et al., 2015).

Dr. Yan-Jiang Wang (The Third Military Medical University) discussed peripheral Aβ clearance in Alzheimer's disease (AD) brains. Dr. Wang discussed available evidence regarding the mechanisms of both endogenous and exogenous Aβ-specific antibodies, with a view to developing optimal immunotherapy based on peripheral Aβ clearance, targeting the toxic domain of Aβ, and improving antibody specificity. Such strategies could improve immunotherapy safety and efficacious disease-modifying treatment options for AD (Liu et al., 2012; Wang et al., 2016).

Dr. Jia-Li Li (Kunming Institute of Zoology, CAS) described recent progress in the study of epigenetic primate brains during development and aging. In their study, comprehensive RNA-Seq analysis was applied to characterize dynamics of lncRNA expression in rhesus macaque brains across postnatal development and aging. A total of 18 anatomically diverse lncRNA modules and 14 mRNA modules representing spatial, age, and sex specificities were identified. Co-expression and negative correlation between lncRNAs and mRNAs were functionally associated with brain development and aging, especially in the neocortex. These findings provide insight into spatial-, age- and sex-related dynamics of lncRNA expression during postnatal brain development and aging in macaques, implying that high dynamics of lncRNA expression might represent a previously unappreciated regulatory mechanism in shaping brain architecture and function (unpublished data).

Drs. Jian-Hong Wang and Gong Chen (KIZ, CAS) presented recent work on the *in vivo* reprogramming of reactive glial cells directly into the functional neurons by single neural transcription factor NeuroD1 in the monkey brain. Stroke is a major and difficult to cure brain disorder. Clinical and experimental studies have struggled to resolve glial scars after stroke. Dr. Chen has led groundbreaking work on reprogramming reactive glial cells directly into neurons in the mouse brain with injury or AD (Guo et al., 2014), leading to a novel method to reverse glial scars to neural tissue. Dr. Chen reported on the chemical reprogramming of human astrocytes into functional neurons with a cocktail of nine small molecules. This chemical reprogramming is mediated through epigenetic silencing of glial genes and transcriptional activation of neural transcription factors such as NeuroD1 and Neurogenin 2 (Zhang et al., 2015). Dr. Chen's team have also successfully established a focal cerebral ischemia model in the rhesus monkey, and successfully expressed the NeuroD1 transcription factor in the ischemic area through infection of adeno-associated virus AAV2/9-NeuroD1. They found that NeuroD1 is expressed in the monkey's cortex and protects the brain after endothelin-1 (ET-1) induced newborn neurons in NeuroD1-infected areas, paving the way for the potential clinical application of this new technology. Implementation of this study suggests a new strategy for the treatment of stroke and other brain disorders.

Stem cell and transgenic NHP models

In the past decade, particularly in the last couple of years, the development of novel genome editing tools (ZFNs, TALENs, CRISPR/Cas9 system) has broadened the possibility of adapting mouse transgenic techniques to NHP.

Dr. Ping Zheng (KIZ, CAS) presented the latest study from her lab on the development of the transgenic tree shrew (*Tupaia belangeri*) model using genetically modified spermatogonial stem cells. Tree shrews have a close relationship to primates and have many advantages over rodents in biomedical research. However, a lack of gene manipulation methods has hindered their wider usage. Dr. Zheng described a culture system for the long-term expansion of tree shrew spermatogonial stem cells (SSCs) without the loss of stem cell properties. The expanded tree shrew SSCs were transfected with enhanced green fluorescent protein (eGFP)-expressing lentiviral vectors. After transplantation into sterilized adult male tree shrew testes, the eGFP-tagged SSCs could restore spermatogenesis and successfully generate transgenic offspring. Thus, the development of a culture system to expand tree shrew SSCs in combination with gene editing paves the way for precise genome manipulations using the tree shrew (Li et al., 2016).

Dr. Yu-Yu Niu (Kunming University of Science and Technology) discussed the development of human disease models in NHPs using gene-editing technology. Dr. Niu discussed NHPs as powerful experimental models to study neurodegenerative human diseases, such as Parkinson's, Alzheimer's, and Huntington's diseases, which occur due to genetic mutations. Because such human diseases do not occur naturally in NHPs, transgenic NHPs need to be established to understand the etiology of disease pathology and pathogenesis. Compared with rodent genetic models, the generation of transgenic NHPs for human diseases is inefficient, and only a few transgenic monkey models have been reported. Dr. Niu's group have focused on the potential approaches and contributing factors for generating transgenic NHPs to study human diseases (Chen et al., 2016a).

Dr. Yue-Jun Chen (Shanghai Institute of Neuroscience, CAS) presented recent work on the chemical control of grafted human pluripotent stem cell (hPSC)-derived neurons in a mouse model of Parkinson's disease (PD). The study showed tunable rescue of motor function in a mouse model of PD, following transplantation of human midbrain dopaminergic (mDA) neurons differentiated from hPSCs engineered to express designer receptors exclusively activated by designer drugs (DREADDs). Administering clozapine-N-oxide (CNO) enabled precise DREADD-dependent stimulation or inhibition of engrafted neurons, revealing D1 receptor-dependent regulation of host neuronal circuitry by engrafted cells. Transplanted cells rescued motor defects, which could be reversed or enhanced by CNO-based control of graft function, and activating engrafted cells drove behavioral changes in transplanted mice. These results highlight the ability to exogenously and noninvasively control and refine therapeutic outcomes following cell transplantation (Chen et al., 2016b).

Dr. Tian-Qing Li (Kunming University of Science and Technology) described recent study on primate pluripotent stem cells and their application in brain science. Dr. Li's group established chimeric monkey animals using embryonic stem cells (ESCs) and showed that cynomolgus monkey ESCs

(cESCs) grown in adjusted culture conditions could be incorporated into host embryos and develop into chimeras with contribution in all three germ layers and in germ cell progenitors. Under optimized culture conditions, which were based on an approach developed previously for naive human ESCs, the cESCs displayed altered growth properties, gene expression profiles, and self-renewal signaling pathways, suggestive of an altered naive-like cell state. These findings show that it is feasible to generate chimeric monkeys using ESCs, thus opening new avenues for the use of NHP models to study both pluripotency and human disease (Chen et al., 2015).

Drs. Zheng-Bo Wang and Xin-Tian Hu (KIZ, CAS) shared their recent work on neurons differentiated from transplanted stem cells, which were shown to respond functionally to acoustic stimuli in the awake monkey brain. They developed a technique in which a small “hole” is created in the inferior colliculus (IC) of rhesus monkeys, with stem cells then transplanted *in situ* to investigate their integration into the auditory neural network. They found that some transplanted cells differentiated into mature neurons and formed synaptic input/output connections with the host neurons. They further verified that the transplanted cells have the potential to functionally integrate into the host neural network (Wei et al., 2016).

Dr. Xu-Dong Zhao (KIZ, CAS) reviewed the importance of primates in biomedical research, especially in preclinical research, immunology, tumor immunology, and immunotherapy. The nonhuman glioblastoma model established by Dr. ZHAO’s lab showed similar histopathology, expression profiles, and image characteristics to those of human patients (unpublished data).

NHP animal welfare and others

The three presentations in this session highlighted NHP research outside of China, as well as ethical and welfare awareness and the application of NHP resources in trans-medical research.

Prof. Eilon Vaadia (Hebrew University, Israel) discussed present and future direction of NHP resources and projects in Israel from the viewpoint of people, work, and outlook. Moreover, the possible collaboration for studies across multiple disciplines between KIZ and his institute were discussed.

Dr. De-Ming Sun (National Health and Family Planning Commission of the PRC) introduced rules for the welfare and ethical implications of using NHP in biomedical experiments. As a representative from NHP breeding enterprises, **Mr. Zheng-Wu Wang (**Sichuan Yibin Hengshu Bio-Technology Co., Ltd.) introduced the status and market demand of nonhuman primate breeding in China, and discussed the possibility of industry-university research cooperation.

Dr. Li Wang (Sichuan University) introduced the urgent need for and challenges in the evaluation of drug efficacy and opportunities of NHP models in the field. Examples for the application of NHP models in human diseases, such as heart failure, neural disorders, and diabetes, in the Chengdu-based drug evaluation center, one of the largest in China, were also presented.

In summary, the organizers of the 4th NHP symposium would like to take this opportunity to express our appreciation and gratitudes to all the attendees for their excellent lectures and active participation in discussions during the meeting. The organizers sincerely hope that the next symposium will continue to flourish both domestically and internationally and offer researchers many opportunities to collaborate and advance innovative research, from basic studies to translational medicine, using NHP resources.

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A clarification of authorship in an old publication

Recently, the editorial office of Zoological Research (ZR) received a statement from Prof. Shu-Yi Zhang regarding his authorship of an old publication (Ma et al., 2003) in ZR (see below). He requested to withdraw his authorship of this article. The editorial office took this statement seriously and the editor-in-chief and associate editors-in-chief had in-depth discussions on this issue. Despite the fact that ZR announces clearly that it copes with the authorship measurements of International Committee of Medical Journal Editors (ICMJE, 2013), and gives guidance to authors regarding the proper authorship in the Instruction to Author, the actual practice still depends on the author himself or herself (Liu, 2016). As a way to clarify the related concerns, ZR decided to publish the clarification provided by Prof. Zhang and the corresponding replies from the other co-authors of this article.

Editorial Office of Zoological Research

Statement from Dr. Shu-Yi Zhang

I write to you regarding the research report entitled "Piscivorous Habit and Echolocation Sound of Myotis ricketti at Fangshan, Beijing" 2003, 24(4): 265-268, of which I'm listed as one of the two co-corresponding authors. When the manuscript was submitted from Beijing to Zoological Research, I was carrying out a long-term investigation on SARS outbreak in Southern China, and was not informed of the manuscript submission. I did not participate in the drafting of this manuscript; therefore, I would like to withdraw my authorship of this article. Otherwise, I will not bear any responsibilities of data or words in this research report.

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Replies from the other authors of this article

The ZR editorial office sent Dr. Shu-Yi Zhang's letter to all authors of this article. Dr. Bing Liang (Institute of Zoology, Chinese Academy of Sciences) and Dr. Gareth Jones (University of Bristol, UK) expressed their support to Dr. Zhang's request of withdrawing his authorship. In his e-mail to ZR editorial office, Dr. Jones wrote "*Professor Zhang's request seems very reasonable to me, and I support it. Because the paper is in Chinese (which I cannot read), I was not aware of it either. I appreciate Dr, Ma's generosity in including me as an author, but because I was unaware of the paper, I should not be included as an author either*".

Dr. Jie Ma (Harvard University), the first author of this article, replied that "*I apologize to those authors who did not read the draft before the submission. Therefore, I would like to fully respect the authors' requests to remove their authorship from this paper*".

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